Insights into the Pathogenesis of Pediatric IBD:

Epithelial Barrier, Immune Dysregulation, and the Gut Environment.

by

Deenaz Zaidi

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Medical Sciences - Pediatrics,
University of Alberta

ABSTRACT

Introduction:

Inflammatory bowel diseases (IBD), encompassing Crohn disease (CD) and ulcerative colitis (UC) are on a steadfast global increase, and in particular, are highly prevalent in children in Canada. Current treatment is targeted towards controlling inflammation and to date no certain etiology or cure have been found. Multiple factors play integral roles in disease pathogenesis, such as, dysfunctional epithelial barrier, altered microbial composition, and inappropriate immune responses, but the mechanism leading to disease is still unknown. In my PhD project, I investigated critical, interlinked factors associated with IBD pathogenesis, *i.e.*, the gut barrier, microbial virulence, cytokines, and factors that control inflammation. The objectives of this thesis were to evaluate gap density in pediatric IBD patients, correlate it with changes in cytokines and inflammatory markers, and to establish its prognostic value; to investigate the role of A20, also known as tumor necrosis factor α-induced protein 3 (TNFAIP3), in IBD pathogenesis; and to study the relationship between microbial virulence, gut environment, and the gut barrier.

Methods:

The experimental approach to address my objectives was achieved through a cohort study of known IBD patients and non-IBD controls between the ages of 3-18 years. The control group included patients undergoing gastroscopy and colonoscopy

for indications other than IBD. Epithelial gap density in the duodenum of recruited patients was evaluated using probe-based confocal laser endomicroscopy (pCLE) with fluorescein injected as contrast during endoscopy. Biopsy samples were obtained from the duodenum and terminal ileum and assessed for A20 and associated factors. Duodenal and terminal ileum aspirates were collected for analyses of bacteria and their function and virulence, using metabolomics and *in vitro* approaches, respectively.

Results:

Epithelial gaps assessed with pCLE were found to be increased in the duodenum of children with both CD and UC but unrelated to inflammation. This suggests that an altered epithelial barrier is an important systemic feature of pediatric IBD and is not only secondary to inflammation. I was also able to utilize pCLE to assess vascular flow in the duodenum of non-IBD and IBD patients and found capillary flow rates to be significantly higher in the duodenum of UC patients.

Results from exploring the role of the TNF-α counteracting protein, A20 in IBD also showed important findings related to a possible mechanism for failure of A20 to down-regulate inflammation in pediatric CD. I found that despite an increase in *A20* expression in TI biopsies of CD patients, the protein levels were low. The discrepancy between *A20* expression and protein levels was possibly due to the concomitant lower expression of ABIN-1, an A20 accessory protein, and instability of the A20 protein due to lack of post-translational phosphorylation, possibly related

to lower expression of IKKβ. *Escherichia coli* strain LF82 augmented *A20* expression, but not A20 protein, suggesting that microbes can hinder the capacity of A20 to limit inflammation. Thus, factors affecting A20 and microbes could negatively impact the protein's anti-inflammatory action in pediatric CD, and contribute towards unremitting inflammation.

Effects of intestinal aspirates on microbial invasion potential were evaluated using Gentamicin protection assays by infecting T-84 and Caco-2 cells with various *E. coli* strains. Invasion potential of some *E. coli* strains was greatly increased in the presence of intestinal aspirates. Metabolomic analysis of intestinal aspirates identified variation in the quantity of metabolites in the intestinal aspirates of non-IBD and IBD patients, which could possibly explain the invasion potential and virulence factors of bacteria.

Conclusion:

This thesis has revealed important information about key factors involved in causing and affecting the course of IBD. I found that epithelial gaps are increased in IBD, and that there is dysregulation in factors controlling inflammation. Findings from this work might help in improving treatment strategies, such as medications that enhance the gut barrier and reduce epithelial cell shedding and drugs that target specific proteins and reduce inflammation. Taken together, the results of this thesis show significant findings that are important contributors to our understanding of

pediatric IBD pathogenesis, and provide a basis for additional scientific work targeted towards a therapeutic approach.

PREFACE

The research presented in this thesis was conducted by Deenaz Zaidi. Ethics approval for approaching the pediatric patients included in this study was obtained from the University of Alberta Research Ethics Board (Study ID Pro00023820). All experimental work and patient recruitment procedures reported in this thesis were under the supervision of Dr. Eytan Wine, the Principal Investigator.

The first objective of my thesis (**Chapter 4**), to analyze epithelial gaps in a cohort of pediatric non-IBD and IBD patients is published as, *'Zaidi D, Bording-Jorgensen M, Huynh HQ, Carroll MW, Turcotte JF, Sergi C, Liu J, Wine E. Increased Epithelial Gap Density in the Non-Inflamed Duodenum of Children with Inflammatory Bowel Diseases*. *J Pediatr Gastrenterol Nutrn.* [published; December, 2016]'. EW and DZ designed the study. EW, HQH, MWC, JL, and JFT conducted the confocal imaging and collected samples. DZ and MBJ analyzed the confocal images for epithelial gaps. CS analyzed the tissue sections for IEL counts. DZ conducted the experiments. DZ and EW wrote the paper; all authors reviewed and approved the final version of the paper.

For **Chapter 5**, confocal imaging was conducted by EW, HQH, MWC, and RP. Vascular flow analysis in confocal images was conducted by LC and DZ. This manuscript has been accepted for publication in *J Pediatr Gastrenterol Nutrn.*

For the research work pertaining to **Chapter 6**, biological specimens during endoscopy were obtained by EW, HQH, and MWC. Experimental procedures were designed by EW, SB, and DZ. All experiments were conducted by DZ.

For **Chapter 7**, intestinal aspirates during endoscopy were obtained by EW, HQH, and MWC. Metabolomic analysis of intestinal aspirates was conducted by Rupa Mandal and Dr. David Wishart of University of Alberta. All other experiments were conducted by DZ.

This work is dedicated to my loving parents,

my best friend Sameen

and

our beautiful daughter Manaal

ACKNOWLEDGEMENTS

This exciting and joyous milestone could not have been achieved without the support of many people and I want to convey my heartfelt gratitude to all of you. I want to thank my best supervisor Dr. Eytan Wine, who gave me the opportunity to work on this project. Thank you for being a great teacher, who was always there with me at every step! You laid the foundation of translational medicine for me, by teaching me to effectively link clinical knowledge with basic sciences. Your mentorship honed my skills and helped me recognize my strengths and weaknesses. You taught me to think outside the box, to develop critical thinking, and most importantly, to expand and build-up on my clinical knowledge beyond traditional care, utilizing science. Your continuous encouragement to explore the unknown, and positive attitude made this journey easy and enjoyable. I will continue to apply what I have learned from you in my future endeavors, both on professional and personal fronts.

I also want to thank my supervisory committee members Dr. Karen Madsen, Dr. Hien Huynh and Dr. Shairaz Baksh for your constant support towards me. Your sound advice, knowledge and honest opinion helped me greatly in all of my projects and kept me on the right track. You are a wonderful team who helped me achieve my goals in a timely and successful manner.

The support that I received from the Department of Pediatrics, The Centre of Excellence for Gastrointestinal Inflammation and Immunity Research (CEGIIR) and Stollery Children's Hospital was tremendous. Thank you, our brave patients, for your active participation that made this work possible. Your strength and willingness amaze me! I want to specially thank Dr. Matthew Carroll, Dr. Julia Liu, Dr. Rabin Persad, Dr. Sujata Persad, Dr. Justine Turner, Dr. Jason Yap and Dr. Leo Dieleman. I really appreciate the support you provided. A huge thanks is for Trish Kryzanowski; you made life easy by taking meticulous care of everything. I would like to thank Dr.

David Wishart for help with metabolomics. I also want to thank the funding support that I received through the Graduate Studentship from WCHRI and the Medical Sciences Graduate Studentship from University of Alberta that greatly helped with my studies.

Special thanks for my lab members, Michael Bording-Jorgensen, Rosica Valcheva, Youfei Lou, Misagh Alipour, Rosanne Govaarts, Lucas Churchill, Joe Ou, Heather Armstrong, and friends in the CEGIIR group. Thank you for your help and for creating a cheerful and fun environment.

Warmest thanks to my parents! Thank you, Ammi and Abba, I am very lucky to be your daughter! Your hard work, the way you raised me and encouragement are instrumental in all that I have achieved in every aspect of my life. Your love and dedication enabled me to achieve my goal of becoming a Doctorx2 and I am very proud of both of you! I would also like to thank Abbas, my brother, for his unconditional support.

Huge thanks to my best friend and better-half Sameen; your love, tremendous support and understanding has always encouraged me to be my very best and none of this would have been possible without you. Special thanks to our daughter Manaal, for your patience and support during my studies! You are our shining star who made all the efforts and hard work worth it!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of abbreviations (in order of appearance):

IBD	Inflammatory bowel diseases
CD	Crohn Disease
UC	Ulcerative colitis
pCLE	Probe-based confocal laser endomicroscopy
EGD	Esophagogastroduodenoscopy
ESR	Erythrocyte sedimentation rate
CRP	C-reactive protein
PCDAI	Pediatric Crohn's Disease Activity Index
PUCAI	Pediatric Ulcerative Colitis Activity Index
IEL	Interstitial epithelial lymphocytes
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells)
PPAR-γ	Peroxisome proliferator activated receptor-γ
REG3γ	Lectin regenerating islet-derived protein 3γ
GWAS	Genome wide association studies
VEGF	Vascular endothelial growth factor
IFN-γ	Interferon gamma
vWF	von Willibrand factor
MAMPs	Microbe-associated molecular patterns
DAMPs	Damage-associated molecular pattern molecules
NEMO	NF-kappa-B essential modulator
NMR	Nuclear magnetic resonance spectroscopy
MS	Mass spectrometry

LC	Liquid Chromatography
TCA	Tricarboxylic acid
Tregs	T regulatory cells

Chapter 1

Introduction

1. INTRODUCTION

1.1 The Gastrointestinal Tract

1.1.1 The Anatomy of the Gastrointestinal Tract

The human gastrointestinal tract is a complex structure where variation in anatomical regions is largely correlated with the function of the organs. The gastrointestinal tract starts at the mouth and ends at the anus, and is lined by multiple distinct layers: mucosa, submucosa, muscularis propria, and serosa. Each of these layers has a distinct structure, described as follows. The mucosa contains 3 sub-layers: the epithelium, the lamina propria (containing lymphatics from the esophagus to the small intestine and immune cells throughout the tract, the highest proportion being in the colorectal region), and the muscularis mucosa (containing circular and longitudinal muscle fibres). The submucosa contains the vascular network and Meissner nerve plexus. The muscularis propria contains the Auerbach nerve plexus and two layers of muscles, the circular and longitudinal muscle layers, encasing the Auerbach plexus between them. The serosal layer is the outermost layer or casing and only lines part of the intestinal tract (Atlas and Text Book of Human Anatomy, McMuricch). Following is a detailed description of each layer, its structure, and function.

Oesophagus:

The main role of the oesophagus is to safely transfer food from the mouth to the stomach. It is divided into a cervical and three thoracic segments (upper, mid, and lower), and is lined with multi-layered, non-keratinizing squamous epithelium. The lower most part of the oesophagus, the lower oesophageal sphincter, connects the oesophagus to the stomach and prevents movement of food back (reflux) from the stomach to the oesophagus.

Stomach:

The stomach contains enzymes and acid that aid in digestion. The stomach is divided into four parts: cardia, fundus, body, and antrum, proximally to distally. The lining of the stomach is divided into longitudinal folds, called rugae, which contain gastric pits (invaginations that pass gastric secretions from the glands to the mucosal surface). Similar to all other parts of the digestive tract, except for the esophagus, the stomach epithelial lining is formed by a single layer of epithelial cells. The surface of the stomach is lined by foveolar cells that secrete mucin, while other cell types line the gastric glands, whose secretions aid in the digestive process. The glands of the body and fundus mainly contain parietal, chief, mucous neck cells, and enteroendocrine cells. Parietal and chief cells lack mucin. The cardiac and antral regions mainly contain mucin-producing cells. In addition, the antrum contains gastrin-producing G cells, as well as somatostatin-producing D cells. The lamina propria of the stomach contains lymphatic and vascular networks, plasma and mast cells, and eosinophils. The muscularis propria of

the stomach contains three smooth muscle layers (including an oblique layer, not found in other parts of the bowel) and the Auerbach plexus. Gastric submucosa is enriched with blood vessels and the Meissner plexus. The gastric serosal layer is lined by flattened cuboidal peritoneal cells, similar to other serosal layers in the gut.

Small Intestine:

The main function of the small intestine is digestion and absorption of nutrients. The small intestine is subdivided into three parts: the duodenum, jejunum, and ileum. The most proximal section, the duodenum, lies mostly in the retroperitoneal space, while the jejunum and ileum are intraperitoneal. The duodenum connects the gastric pylorus to the jejunum. It is subdivided into the duodenal bulb, descending, horizontal, and ascending parts. Distal to the ligament of Treitz, the first third of the intraperitoneal small intestine forms the jejunum, followed by the narrower ileum, forming the distal two-thirds of the intraperitoneal small intestine.

The surface of the small intestine is marked by villi and plicae circulares. These unique features greatly increase the surface area of the small intestine to 200-500m^2, which helps increasing the absorptive capacity of the organ. Crypts of Lieberkuhn are invaginations of the epithelium that penetrate down to the muscularis mucosa. The intestinal stem cell niche is the source of intestinal cell lineage and renewal. Present at the base of the crypt, the intestinal stem cell niche contains pluripotent intestinal stem cells that terminally differentiate into intestinal cell lineages. The cells lining the epithelium are mostly columnar. There are basically four types of epithelial cells in the small

intestine: enterocytes, enteroendocrine cells, Paneth cells, and goblet cells. As the cells mature, they migrate up the crypt-villus axis and are ultimately shed into the lumen, except for Paneth cells that remain at the base of the crypts (Peterson & Artis 2014a). Enterocytes are lined by a brush-border consisting of microvilli on the apical side and are mainly responsible for nutrient absorption. Goblet cells (shaped as goblets) contain large amounts of acid-mucin, whereas Paneth cells have antimicrobial proteins and growth factors in eosinophilic granules and are present at the base of the crypts. The lamina propria consists of a capillary network, lacteals (at villous tips), plasma cells, and lymphocytes. The muscularis mucosa contains inner and outer smooth muscle layers. The submucosa contains Meissner plexus, blood and lymphatic vessels, and Peyer patches. Peyer patches are aggregates of lymphoid follicles. The epithelium above Peyer patches contains many intraepithelial lymphocytes (IELs). A specialized type of epithelial cells, called M (microfold) cells, act as mediators between the lymphoid system and IELs and allow for sampling of microbial contents. In addition, specialized mucin-producing glands, Brunner glands, are present in the duodenal submucosa. Muscularis propria is composed of outer longitudinal and inner circular layers that contain the Auerbach plexus in between them. Anteriorly, the duodenum is covered by serosa, and posteriorly, by a connective tissue layer, the adventitia. The jejunum and ileum are covered by serosa.

Colon:

The colon is anatomically subdivided (from proximal to distal) into the cecum, ascending, transverse, and descending colon, followed by the sigmoid colon and rectum.

The main function of the colon is reabsorption of fluid and electrolytes. The colonic anatomy is marked by plica semilunaris, which are transverse mucosal folds with haustral sacs in between. Microscopically, the colonic epithelium is arranged into crypts and does not have villi. The crypt epithelium mostly consists of mucin-containing goblet cells, along with some absorptive cells, and abundant IELs. Enteroendocrine cells present in the small bowel are present in the base of the crypts of all parts of the colon as well. The lamina propria of the colon contains inflammatory cells but is devoid of lymphatic vessels. The concentration of inflammatory cells (lymphocytes, plasma cells, eosinophils) is higher in the ascending colon than other colonic areas. The muscularis mucosa consists of inner circular and outer longitudinal layers, and the submucosa includes the Meissner plexus. The muscularis propria has inner circular and outer longitudinal smooth muscle layers, which are arranged in three bundles, the taeniae mesocolica, taeniae ometalis, and taeniae libera, collectively called the taeniae coli. The taeniae coli are the portal of entry for blood vessels in the colon.

The crypts in the rectum are shorter than those found in the rest of the colon and are widely spaced. Rectal lamina propria has a large number of inflammatory cells and muciphages (mucoprotein containing macrophages) (Lammert 2014).

1.1.2 Structure and Function of Intestinal Epithelium

One of the major functions of the human gut is selective absorption of nutrients as well as preventing the entry of pathogens and antigens from the lumen to the underlying

mucosal tissues. This important physiological function is achieved via a highly selective regulator, the intestinal epithelium. The intestinal epithelium is composed of a single layer of columnar epithelial cells that separate the intestinal lumen from the underlying tissue. About 80% of intestinal epithelial cells are enterocytes, whose main function is absorption. Different types of cells are present in the intestine and have various respective functions. Secretory intestinal cells consist of three types of cells: enterocytes that are involved in absorption, goblet cells that secrete mucin, and Paneth cells that secrete antimicrobial peptides (AMPs). The fourth type, enteroendocrine cells, secrete hormone regulators for the gastrointestinal tract (van der Flier & Clevers 2009).

Epithelial cells are connected to each other by intercellular junctional complexes. The junctional complexes are composed of tight junctions (TJ) and adherens junctions (collectively known as the apical junctional complex), desmosomes, and gap junctions. Intercellular junctional complexes contribute significantly towards maintaining a stable state for epithelial permeability. Adherens junctions, gap junctions, and desmosomes are also involved in intracellular communication and cell-cell signalling (Perez-Moreno & Fuchs 2006; Garrod & Chidgey 2008; Sosinsky & Nicholson 2005). Tight junctions act as a selective and regulated seal between the apical and basolateral aspects of epithelial cells, allowing certain molecules to pass through, while preventing others and bacteria from entering the underlying tissue.

Membrane proteins form an integral part of the TJ complex. The proteins associated with tight junctions include claudins (Tsukita et al. 2001), occludin (Furuse et al. 1993), junctional adhesion molecule (Martìn-Padura et al. 1998), and tricellulin

(Ikenouchi et al. 2005). Subtypes of claudins have been found to be associated with various regulatory functions within the gastrointestinal membrane layer. Claudins 1, -3, -4, -5, and -8 act as barrier sealants (Milatz et al. 2010; Van Itallie et al. 2001; Amasheh et al. 2005; Amasheh et al. 2009; Furuse et al. 2002). Claudins 2, -10b, and -15 act as channels that allow passage of selective ions (Tamura et al. 2011; Amasheh et al. 2002; Günzel et al. 2009).

An intact intestinal epithelium acts as a mechanical barrier that safeguards against many diseases such as, IBD (Suenaert et al. 2002), type 1 diabetes (Damci et al. 2003) and celiac disease (Schumann et al. 2012), all of which are characterized by a leaky intestinal barrier. Thus, the integrity of the intestinal epithelial lining, as well as stable cell junctional complexes and the proteins associated with them, are important contributors to normal gut barrier function and structural stability.

1.1.3 The Gut Barrier and Immune Homeostasis

Gut homeostatic balance is key towards maintaining physiological harmony between the contents of the gut lumen and underlying tissue, in order to maintain a healthy environment. The gut barrier is composed of several elements: commensal bacteria, the mucus layer, epithelial lining, immune cells, vasculature, and muscle layers that collectively form a 'biophysical' barrier that provides protection against pathogens and other potentially harmful luminal factors.

The first barrier in the lumen that invading organisms have to conquer consists of the commensal bacteria that normally populate the gut. Resident commensal microbes of the gut act as sentinels against pathogens and significantly impact and shape immune responses (Buffie & Pamer 2013). Commensals exert a mechanism called 'colonization resistance'. This term was coined as commensals carve specific niches in the gut that prevent pathogens from accessing nutrients required for their nourishment and growth through competition (Buffie & Pamer 2013; Martinez-Medina et al. 2009). Antimicrobial factors, and microbe-microbe killing are additional protective mechanisms. Gut motility and pH also contribute to the gut barrier.

After the first point of contact with commensals, the next barrier that the invading organisms need to face is the mucus layer. Although the mucus layer is present throughout the gut, the distribution and morphology vary throughout the gastrointestinal tract. In the oesophagus, the mucus layer is patchy, whereas, it is more continuous in the stomach (Johansson et al. 2013). In the small intestine, the mucus layer is patchy (discontinuous), whereas, in the colon there is a consistent outer loose and inner dense mucus layer. The mucus layer is composed of secreted and transmembrane mucins that form a physical barrier in the gut; this bars direct contact between epithelial cells and gut luminal contents. Mucins are highly glycosylated proteins that are secreted by glands and mainly goblet cells in the gastrointestinal tract. The importance of the mucus in gut protection is evident by the fact that Mucin 2 oligomeric mucus gel-forming (MUC-2) deficient mice are prone to developing colitis and colorectal cancer (Van der Sluis et al. 2006) and the phenotype of the colitis resembled human UC (Wenzel et al. 2014).

Intestinal epithelial cells do more than form a physical barrier between luminal contents and underlying tissue; they also provide biochemical and innate immune protection against pathogens (Cerovic et al. 2012; del Rio et al. 2010). Epithelial cells, through pattern recognition receptors (PRR), sense microbial motifs and modulate host immunity accordingly, as observed in studies where deletions in the Toll-like receptor (TLR) signalling pathways, myeloid differentiation primary response gene 88 (MyD88), or inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), (Nenci et al. 2007) led to colonic inflammation in mice. There is a continuous cycle of renewal of epithelial cells as they migrate up the crypts, shed into the intestinal lumen, and are replaced by new cells. The physiology and pathophysiology of intestinal cell shedding is discussed below in section 1.2.3.

Antimicrobial peptides (AMPs) also play a significant role in preventing infection, which can lead to inflammation. For example, enterocytes secrete C-type lectin regenerating islet-derived protein IIIγ (REGIIIγ) in the small intestine and colon, and Paneth cells secrete lysozyme, defensins, and cathelicidins in the small intestine (Dupont et al. 2015). Of these AMPs, defensins and cathelicidins target bacterial surface membranes and form pores to destroy them (Gallo & Hooper 2012). Mouse models have also shown that mucus and AMPs act in conjunction to prevent pathogens from crossing the gut barrier (Cobo et al. 2015; Pelaseyed et al. 2014).

'Antigen sampling', a phenomenon manifested by intestinal microfold 'M' cells, leads to immunogenic responses in the intestine. Antigen sampling by dendritic cells also contributes to tolerance in the intestine (Mabbott et al. 2013). An important role of

commensal bacteria towards maintaining a stable gut barrier in a colitis mouse model has been suggested through intestinal cell deficient MyD88 and TLR deficient mouse models that become ill when they are treated with antibiotics thus supporting their contribution to intestinal homeostasis (Rakoff-Nahoum et al. 2004).

The immune system of the gastrointestinal tract is a complex, well-orchestrated network that remains active throughout life to maintain a steady state of health and homeostasis. The gut environment is constantly exposed to different exogenous materials, including pathogens. A major challenge faced by the intestinal immune system is to effectively eradicate pathogens while ensuring survival of beneficial gut commensals at the same time. Various cytokines are involved in both CD and UC, (Neurath 2014a), as well as disruptions in the NF-κβ (Nuclear factor kappa-light-chain-enhancer of activated B cells) inhibitory pathways, such as the *A20* (Tumor necrosis factor α-induced protein 3) gene (Atreya et al. 2008; Ma & Malynn 2012). The role of A20 is further described in section 1.3.2.

Host-microbial interactions are major drivers of immune homeostasis and disease. The gut immune system develops a state of 'tolerance' towards resident microbes to ensure their survival. Commensals provide signals to the immune system to establish and maintain an adequate anti-inflammatory response. Gut commensals also regulate anti-inflammatory activity in the gut. An example is that of *Bacteroides thetaiotaomicron* that inhibits NF-κβ activity by affecting peroxisome proliferator activated receptor-γ (PPAR-γ); this then diminishes the NF-κβ subunit RelA from being transported into the nucleus (Kelly et al. 2004). The recognition of commensals by TLRs, and subsequent

immunomodulatory changes, play significant roles in this host-microbial harmony, as observed in germ-free mice. Inoculation of germ-free mice with *Bacteroides fragilis* led to induction of IL-10-producing Foxp3(+) T regulatory cells (Tregs) (Balzola et al. 2010). Induction of TLR and Paneth cell signalling by commensals result in the production of REGIIIγ (Vaishnava et al. 2008). Probiotics, defined as 'live organisms that are beneficial to the health of the host when administered in sufficient quantities' (FAO & WHO, 2001), have been shown to enhance barrier integrity in mouse models of colitis (Madsen 2012) and exert a reduction in intestinal permeability in pediatric IBD patients (Gupta et al. 2000). In the submucosal tissue, PRRs also contribute to 'tolerance', as mice in which TRAF-6 was deleted in dendritic cells had an increased number of T helper (T_h) 2 cells along with development of enteritis and depleted Tregs. Interestingly, this loss of tolerance response occurred only in the presence of gut commensals (Han et al. 2013).

1.2 Inflammatory Bowel Diseases

1.2.1 Overview of Inflammatory Bowel Diseases

Inflammatory Bowel Diseases (IBD) are chronic debilitating disorders of the digestive system with significant long term complications. Largely subdivided into two main subgroups, Crohn disease (CD) and ulcerative colitis (UC), the incidence and prevalence of these idiopathic inflammatory disorders of the gut are increasing globally over the last few decades. The median age of onset is between 15-30 years. CD can

involve any part of the gastrointestinal tract, characterized by 'skip' lesions, whereas, UC almost exclusively affects the colorectal region in a diffuse continuous pattern (Zhang & Li 2014). In UC, the lesions are confined to the mucosa, whereas, CD is characterized by transmural involvement (Abraham & Cho 2009). Common symptoms of IBD include diarrhea that may contain blood, weight loss, abdominal pain, fatigue, etc. The socioeconomic burden associated with treatment and support system costs for IBD are tremendous. With approximately 233,000 Canadians suffering from IBD in 2012, the costs associated with IBD treatment exceeded 2 billion $ per annum (Rocchi et al. 2012).

1.2.2 Spectrum of Pediatric Inflammatory Bowel Diseases

Along with adults, IBD have greatly impacted the pediatric population as well. The incidence of pediatric IBD is on a steadfast global rise, as reported in a recent systematic review (Benchimol et al. 2011); in fact, about 25% of cases are diagnosed in children. Furthermore, pediatric IBD has been shown to have increased in Ontario, Canada, by analysis of a retrospective cohort of patients diagnosed between 2009-2014, with the incidence increasing from 9.4 to 13/100,000 children (Benchimol et al. 2014). This is in concordance with previous studies that had also stated a steadfast rise (Abramson et al. 2010; Bernstein et al. 2006). Interestingly, the incidence of IBD in Canadian children who have immigrated from other countries has also increased (Benchimol et al. 2015). While IBD has been largely reported to be a disease of

developed countries, there is now evidence of increased disease prevalence in Asian countries (Cakir et al. 2015).

1.2.3 Etiological Associations of Inflammatory Bowel Diseases:

There is not a single cause of IBD; rather, a multitude of factors have been associated with this disease spectrum, such as, microbiota, genetics, gut barrier, and environment (Neuman & Nanau 2012), and are described in detail below.

Genetics:

The advent of genome wide association studies (GWAS) has identified many genetic loci associated with IBD. Meta analysis of GWAS reported 163 loci that are associated with IBD, of which 110 overlapped between CD and UC, and 30 and 23 were uniquely associated with either CD or UC, respectively (Jostins et al. 2012). Recently, 38 new loci have been identified in a trans-ethnic cohort study of European and Asian patients (Liu et al. 2015). However, the total impact of genes on IBD is still limited as genes likely only explain about 20% of the heritable variance (Franke et al. 2010).

Most genes associated with IBD point towards the important role of microbes as these genes are involved in microbial recognition or response to microbes. *NOD2* (nucleotide-binding, oligomerization-domain-containing protein 2), an important gene involved in immune activation (through recognition of bacterial peptidoglycan), is highly associated with CD, along with other mutations in its signalling pathway genes, such as,

RIPK2 (Jostins et al. 2012). Genes associated with the gut epithelial barrier have also been recognized as high risk loci, such as, *MUC-2* (Mucin 2, oligomeric mucin/gel forming) gene, related to the protective mucous layer (Shan et al. 2013). Cadherin Type-1 (*CDH1*), encoding E-cadherin, is an important protein of the adherens junctions. UC-like symptoms were observed with E-cadherin deficiency in DSS colitis mouse models (Grill et al. 2015). Other epithelial barrier genes, such as, Laminin beta 1 (*LAMB 1*), are also associated with UC (Sarlos et al. 2014). Mutations in *ATG16L1* (autophagy-related 16-like 1) are associated with CD, presenting with morphological abnormalities in goblet cells in mice, and Paneth and goblet cell abnormalities in humans (Lassen et al. 2014). The fucosyltransferase-2 (*FUT-2*) gene that encodes fucosyltransferase, which synthesizes H antigen, essential for intestinal bacteria as a carbon source, is also amongst the high risk group (Tong et al. 2014). A20, also known as, tumor necrosis factor α-induced protein 3 (TNFAIP3), is a cytoplasmic protein that acts as a significant inhibitor of NF-κβ-induced inflammation. A strong linkage between A20 and IBD has been established through GWAS studies (Ma & Malynn 2012). The dysregulation of A20 in IBD is the main focus of **Chapter 6**.

Exposome:

Environmental influence, reflected over an individual's life as the exposome, is considered to be a top contributor of IBD, as the progress of disease incidence is linearly associated with industrialization in developed countries and since most disease variance is not explained by genetics. Many environmental factors have been associated with IBD,

such as, smoking, microbes, diet, geographical variation, antibiotics, breastfeeding, etc. (Frolkis et al. 2013). In addition, epigenetic changes, such as, histone alterations and DNA methylation that are linked with IBD are possibly caused by environmental changes (Legaki & Gazouli 2016). There has been much speculation about the 'hygiene hypothesis' being the cause behind IBD. As per the 'hygiene hypothesis', improved sanitary and hygienic conditions limit the chance of infections in childhood that in turn can hinder appropriate immune system development and can also lead to microbial dysbiosis and altered immune regulation. This seems to hold true for developing countries, or in cases where people have migrated from less to more developed regions (Leong et al. 2016).

Gut Barrier:

The epithelial barrier has a critical role in gut homeostasis, as discussed above, and is greatly impacted in IBD. Increased gut permeability was observed in animal models of IL-10 knockouts (Madsen et al. 1999), as well as in IBD patients (Bruewer et al. 2006; Weber & Turner 2007). Interestingly, increased permeability was present even in unaffected relatives of CD patients, which could possibly be linked with mutations in the *NOD2* gene, or could have occurred due to subclinical mucosal inflammation (Teshima et al. 2012). Incubation of non-inflamed gut tissue from CD patients with sodium carpate showed a marked increase in tight junction permeability as compared to controls, increasing the evidence that inflammatory insult does cause a more pronounced change in paracellular permeability in tissue of CD patients (Söderholm et al. 2002). Thus, whether

changes in the gut barrier occur as a consequence of inflammation of IBD, or whether epithelial barrier changes cause IBD, remains to be determined.

Microbial Dysbiosis:

Microbial dysbiosis (an ill-defined term or concept that relates to changes in microbial composition and functions associated with disease) is another salient feature of IBD. There are a large number of microorganisms, collectively called microbiota, that exist in the human body, including bacteria, archaea, unicellular eukaryotes, and viruses (Morgan et al. 2013; Cho & Blaser 2012). Bacteria constitute a major part of our microbiota. Previously, it has been reported that out of the vast population of the microbiota, there are about 10^{14} bacterial cells in the human body, mainly concentrated in the gut, and that these outnumber human cells by a ratio of 10:1 (Turnbaugh et al. 2007). However, a recent analysis has suggested that the actual ratio of human to bacterial cells is closer to 1:1 (Sender et al. 2016). The dominant phyla that exist in the human body are *Bacteroidetes* and *Firmicutes*. The other major phyla that inhabit the gastrointestinal tract are *Fusobacteria, Proteobacteria, Actinobacteria, Verrucomicrobia,* and *Cyanobacteria* (Eckburg et al. 2005). Within the gastrointestinal tract, the colon is the most heavily bacteria inhabited organ, with an estimated population of 70% of the bacteria colonizing the entire body.

Various studies have reported alterations in gut microbial composition in IBD (Dicksved et al. 2008). While each study seems to identify different changes in microbes in IBD, some themes are apparent. Adherent bacteria are increased overall in CD,

especially *E. coli* strains. *Peptostreptococcus, Bacteroides,* and *Eubacteria* are also increased in CD, whereas *Bifidobacteria* are reduced (Linskens et al. 2001). Facultative anaerobes are increased in UC. Another study reported that *Firmicutes* and *Bacteroidetes* are decreased in IBD patients, whereas *Proteobacteria* is increased (Frank et al. 2007). Microbial dysbiosis in disease settings, such as IBD, has been associated with increased epithelial permeability, epithelial dysfunction, and increased stimulation of the immune system but this association is again challenged by poor data supporting a causative relationship (Sartor 2008; Nagalingam & Lynch 2012; Comito & Romano 2012).

1.2.4 Intestinal Epithelial Shedding in Inflammatory Bowel Diseases:

The intestinal epithelial stem cell niche is present in the base of the crypts, where pluripotent stem cells give rise to different lineages of intestinal cells. Mature cells, excluding Paneth cells, migrate up the crypts to the tips of the villi, in the small bowel (Peterson & Artis 2014b). Cells are naturally shed from the intestinal epithelium, at the tips of the villi in the small bowel into the lumen as a normal physiological process, and a balance between epithelial cell shedding and cell proliferation is necessary to maintain a healthy dynamic intestinal epithelium (van der Flier & Clevers 2009). The spaces left in the epithelium after the extrusion of epithelial cells are called 'epithelial gaps'. Epithelial cell shedding has been studied with different techniques, such as with electron microscopy, which resulted in the term 'zipper theory', where an extruding cell is replaced from beneath by extensions from neighbouring cells, along with projections of TJ that occur between the adjacent and the shedding cell along its basolateral margins. This

phenomenon fills the gap and prevents a permanent breach in the intestinal epithelium (Madara 1990). Confocal microscopy in mice showed that the TJ protein ZO-1 is redistributed early in an initial event of epithelial cell shedding (Turner et al. 2011; Marchiando et al. 2011).

Epithelial gap density is defined as the number of gaps per 1000 epithelial cells. Epithelial cell shedding can be visualized *in vivo* during endoscopy, at a microscopic level, using probe-based confocal endomicroscopy (pCLE); this is the focus of **Chapters 3 & 4**. Confocal endomicroscopy was successfully used in mice to visualize and study the mechanisms of epithelial cell shedding and gut barrier integrity (Watson et al. 2005; Watson et al. 2009). Studies done later also showed the utility of this optical imaging technique for high resolution, real-time, live microscopy of the human gut during endoscopy (Liu et al. 2011; Kiesslich et al. 2007; Kiesslich et al. 2012). Epithelial gap density has been found to be increased in adult IBD patients (Liu et al. 2011) and is predictive of disease course (Turcotte et al. 2012). pCLE determined that gap density was related to hospitalization in adult CD patients (Liu et al. 2011). Recently, epithelial gaps analyzed with pCLE were found to be higher in the duodenum of adult IBD patients than in healthy controls (Lim et al. 2014a).

In vitro cell line models using T-84 cells demonstrated that epithelial shedding was induced by caspase-1, which also caused increased cell shedding in mice and altered epithelial permeability in T84 cells. Analysis of human ileum biopsies from IBD patients reported caspase-1-mediated apoptosis to be a possible mechanism involved in epithelial cell shedding (Liu et al. 2013).

TNF-α has been linked with both UC and CD (Sarlos et al. 2014). Incubation of T-84 epithelial cells with TNF-α caused dislocation of occludin-1 and claudin-1 and -4 (Bruewer et al. 2003). Cytokines, especially TNF-α, have been shown to be important regulators of gut barrier function (Bruewer et al. 2003). TNF-α is also involved in intestinal epithelial cell shedding (Watson & Hughes 2012).

1.2.5 Vascular Factors Associated with Pediatric IBD

Vascular changes and endothelial dysfunction are well-characterized features of IBD (Cromer et al. 2011). Vascular endothelial growth factor (VEGF)-A and B induce angiogenesis in IBD (Scaldaferri et al. 2009). Changes in endothelial cells occur in response to inflammation, as various cytokines, such as TNF-α, induce VCAM-1 and MAdCAM-1 and promote platelets adherence in IBD. Degradation of endothelial junctions can be mediated by leukocytes and neutrophils through enhanced protease secretion (Cromer et al. 2011). Endothelial cells form a selective barrier, which is disrupted in IBD. As IBD advances, inflammation induced by IFN-γ (interferon gamma), and epithelial barrier disruption allow microbes to induce microvascular permeability in mouse models (Oshima et al. 2001a). Endothelial dysfunction in UC is more wide-spread than localized, and is positively correlated with disease severity and more pronounced in UC patients with extraintestinal complications than in those with localized lesions (Kocaman et al. 2006).

The gut microenvironment alters many physiological aspects of the microvascular endothelium. Human intestinal mucosal microvascular endothelial cells (HIMECS) isolated from IBD patients demonstrated increased leukocyte binding capacity compared to HIMECS from non-IBD mucosa (Binion et al. 1997). The intestinal microenvironment also affects the endothelium, with inflammation affecting gene expression, such as the *vWF* (*von Willibrand factor)* gene (Aird et al. 1997). Many vascular changes occur due to inflammation; for example, cytokine-associated activation of endothelium leads to leukocyte recruitment by cell adhesion molecules (CAMs) and chemokines (Hatoum et al. 2006). Cytokines involved in IBD also affect gut vasculature, as TNF-α and IL-8 both possess pro-angiogenic properties (Cromer et al. 2011). Neutrophils, VEGF, and histamine can break down endothelial cell to cell junctions (Kumar et al. 2009). Thus, the endothelium acts as a defensive barrier and factors affecting vascular pathology in IBD need to be investigated in more detail. This is the focus of **Chapter 5** of this thesis.

1.2.6 Treatment Regimens for Pediatric IBD

Current IBD therapy is aimed at sustaining immunosuppression, but a definitive cure has not been found yet. The aim of IBD treatment is to induce remission first and then to maintain it, with the ultimate goals of preventing the occurrence of flares, while allowing for normal growth and development, normal function and quality of life, and prevention of long-term complications from the disease or treatments. The balance between drug efficacy and safety is a major therapeutic challenge, as many medications

have the potency to cause adverse effects, as described below in this section.

Sulfasalazine and 5-ASA medications (Asacol, Mesevent, Pentasa) are used to reduce inflammation in UC and have minor and infrequent side effects, such as, rash, headache, and diarrhea. While they are generally considered safe, they are rarely effective in severe and recurrent UC and are ineffective in CD.

Prednisone and Budesonide are oral corticosteroids used to decrease inflammation (typically only during acute flares as induction medications) and are relatively effective in improving symptoms; however, their long-term complications, such as hypertension, osteopenia, high susceptibility to infections, and glucose intolerance render them to be used cautiously (Katz 2004). In children, steroids negatively impact growth and bone health, and cause weight gain, increased appetite, acne, striae, mood swings, bone loss, in addition to concerns mentioned above.

Immunomodulators, such as the thiopurine anti-metabolites, 6-mercaptopurine and azathioprine, have also been a mainstay of IBD therapy. Both drugs have the ability to suppress inflammation, however, not without concerns of side effects, such as, bone marrow suppression, hepatitis, pancreatitis, and rarely, non-melanoma skin cancer and non-Hodgkin's and hepatosplenic T cell lymphoma (specifically associated with use in young males) (Camus et al. 2013; Kotlyar et al. 2011).

Methotrexate is another ‘entry level’ immunomodulator used as a maintenance therapy in CD, or as a combination therapy (similar to thiopurines) with biologics in both CD and UC. Methotrexate interrupts DNA, RNA, and purine synthesis. It too is not without potential toxic effects, such as, increased risk of infections, hypersensitivity,

pneumonitis, and liver fibrosis. Though traditionally not used as frequently as thiopurines, its use has increased recently, especially for young male patients (Alfadhli et al. 2005).

'Biologics', such as infliximab, adalimumab, certolizumab pegol [all of these are anti-TNF-α monoclonal antibodies (MAb)], ustekinumab (anti-IL12/23 MAb), and vedolizumab (anti-integrin MAb) are used for CD and UC. A review of studies reporting the use of biologics revealed that infliximab and adalimumab allow for mucosal healing (Côté-Daigneault et al. 2015) and both can be used as a maintenance therapy, but infliximab is more effective when given with a concomitant immunomodulator. Over the last few years anti-TNF-α agents have become part of mainstream IBD therapy, but not without potential complications, such as increased risk of infection after surgery (Rosenfeld et al. 2013; Kopylov et al. 2012), hepatitis, reactivation of latent tuberculosis, and infections (Lahad & Weiss 2015); however, the benefits, such as continuous mucosal healing, remission, and improved bone density (Griffin et al. 2015) need to be considered against the potential risks and in most cases outweigh them.

Metronidazole and ciprofloxacin are the two antibiotics most commonly used in IBD, however, their efficacy is low and the incidence of flares is high in CD patients after stopping treatment.

Recently, exclusive enteral nutrition (EEN) has come into focus, and has been shown to be just as efficient in inducing remission in pediatric CD patients as corticosteroids, but without any side effects (Soo et al. 2013; Day & Burgess 2013). EEN is also associated with high rates of mucosal healing when used in children with CD

(which steroids do not achieve) and improves bone health (Day & Lopez 2015). The main challenge with this treatment is that it is difficult to complete, since children are restricted to a liquid formula diet for 6-12 weeks (Lahad & Weiss 2015).

Since microbial dysbiosis is a major alteration that occurs in IBD, recent focus on future targeted microbe-altering therapies is heavily under debate (Wine 2014). Probiotics are not effective for CD (Bousvaros et al. 2005). As well, fecal microbial transplant (FMT) has been used as a treatment for IBD, but with variable results across different studies (Colman & Rubin 2014; Moayyedi et al. 2015).

1.3 Tumor necrosis factor α-induced protein 3 (TNFAIP3/A20)

1.3.1 The Role of NF-κβ (Nuclear factor kappa-light-chain-enhancer of activated B cells) in Intestinal Immunity

The gut immune system is tightly regulated in order to prevent an exaggerated inflammatory response to resident commensals, while still retaining the capability to simultaneously activate immune pathways to destroy/control pathogens. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ), a nuclear transcription factor, plays a central role in innate immunity and imbalances in this pathway lead to chronic inflammation and dysregulation in immune responses (Oeckinghaus et al. 2011; Hayden & Ghosh 2008). A pathological shift in gut homeostasis in IBD activates NF-κβ, further propagating inflammation (Atreya et al. 2008). The NF-κβ pathway is activated by various stimuli, such as viruses, bacteria, microbe-associated molecular patterns

(MAMPs), cytokines, damage-associated molecular pattern molecules (DAMPs), ischemia, and oxidative stress (Oeckinghaus et al. 2011).

NF-κβ is composed of several protein subunits: p52, p50, RelA (p65), RelB, and c-Rel, which undergo a process of dimerization in order to regulate gene transcription. The subunits share a common domain, the N-terminal Rel homology domain (RHD), necessary for dimer formation. In the resting state, the subunits are coalesced in the cytoplasm, remaining in the inactive state as long as they are bound to Iκβ proteins. The RelA, RelB, and c-Rel subunits can regulate gene transcription, as they contain a transcriptional activation domain (TAD). The p50 and p52 subunits are capable of binding to DNA, but their transcription-inducing capability is dependent on binding with a protein containing a TAD, such as, RelA, RelB, and c-Rel (Mechtler et al. 2015).

The IKK complex is the key activator of NF-κβ pathway. It is composed of the kinases IKKα and IKKβ and the non-catalytic protein, IKKγ. Activation of NF-κβ requires phosphorylation of Iκβ by IKK that leads to its proteasomal degradation. This releases NF-κβ and allows translocation of the NF-κβ subunits into the nucleus, hence enabling gene transcription.

Activation of the NF-κβ pathway can occur via either the canonical or the non-canonical pathways. The canonical pathway is initiated by receptor-ligand binding, such as stimulation and binding of TLRs with an antigen that results in recruitment of adaptors, such as TRAF 2, TRADD, cIAP1, and cIAP2, with RIP1, to the cytoplasmic domain of the receptor. Additional mechanisms increase the capability of the IKK complex to recruit NF-κβ to the activated receptor, such as K63-linked polyubiquitination

of RIP1. This results in activation of the IKK complex, which phosphorylates Iκβ, leading to its degradation (Ruland 2011).

The non-canonical pathway is dependent on the activation of NF-κB inducing kinase (NIK), which phosphorylates and hence activates the IKKα complex. IKKα then phosphorylates p100, resulting in the production of the p52/RelB active heterodimer and its translocation to the nucleus. The non-canonical pathway is induced by many factors, such as lymphotoxin-α, BAFF, and IL-1β. The turn-off inhibitory signal for NF-κβ is mediated by IκBα, which terminates the inflammatory response by binding to the nuclear NF-κβ complexes and shuttling them back into the cytoplasm (Oeckinghaus et al. 2011).

1.3.2 Mechanism of Action and Regulation of A20

A20 is an NF-κβ-responsive gene that is induced upon NF-κβ activation and encodes the protein A20, also known as tumor necrosis factor α-induced protein 3 (TNFAIP3). A20 is the focus of **Chapter 6**. A20 protein is a cytoplasmic zinc finger protein, which acts as a negative regulator of the NF-κβ signal and an anti-apoptotic mediator (Coornaert et al. 2009). *A20* was originally discovered in 1990 in human umbilical vein endothelial cells as a TNF-α responsive gene (Dixit et al. 1990). Since then, additional research has shown that A20 also counteracts NF-κβ-mediated inflammation that occurs through pattern recognition receptors, CD40, and IL-1 (Beyaert et al. 2000).

A20 regulates the anti-inflammatory pathway in multiple ways; it binds to ubiquitin chains, acting as an E3 ligase, assisting E1 and E2 proteins to build ubiquitin chains; A20 also binds to polyubiquitin chains via its Znf4 domain and deubiquitinates ubiquitin chains (Ma & Malynn 2012). It exerts deubiquitinating properties in order to down-regulate NF-κβ-induced inflammation through its ubiquitin-editing domains. The N-terminal of A20 includes a deubiquitinating (DUB) enzyme domain, consistent of the cysteine protease of the OTU (ovarian tumour) family. A20 removes polyubiquitin chains from the adaptor molecule RIP-1 in TNFR-1 signalling, preventing RIP-1 from interacting with NF-κβ essential modulator (NEMO) and thus inhibits NF-κβ signalling (Wertz et al. 2004). The carboxy-terminus of A20, consisting of seven zinc fingers, acts as a ubiquitin ligase and causes K48-linked polyubiquitination of RIP, leading to its proteasomal degradation (Wertz et al. 2004). In addition, A20 breaks Lys63 (K63)-linked polyubiquitin chains on TRAF-6, which prevents the assembly of the IKK complex, and hence inhibits NF-κβ inflammation triggered by TNF-α.

The stability of A20 is markedly impacted by post-translational modifications. IKKβ is a kinase, which is an important gatekeeper of inflammation. While IKKβ is required to activate the NF-κβ pathway, the protein has also been shown to phosphorylate A20 at the serine 381 (S381) site, which helps A20 stabilize and attenuate NF-κβ signalling. IKKβ-mediated phosphorylation of A2 occurs in response to LPS and TNF (Hutti et al. 2007). Studies in mice with mutations in the A20 zinc-finger-4 (ZnF-4) ubiquitin binding motif or in the ovarian tumor (OTU)-type deubiquitinase domain have shown that A20 in the phosphorylated state cleaves Lys-63-linked polyubiquitin chains

by OTU domain and increases substrate ubiquitination through ZnF-4 (Wertz et al. 2015). This suggests that post-translation modifications of A20, such as phosphorylation, stabilizes A20 and helps it mediate its anti-inflammatory action. The stability of A20 is affected by several other factors, such as the paracaspase MALT-1, which directs T cell receptor signalling to NF-κβ and is a critical factor for T cell activation. Upon T cell receptor stimulation, A20 is directed to a complex of MALT-1 and Bcl-10 that result in cleavage of A20, rendering it unable to attenuate the NF-κβ signal (Coornaert et al. 2008).

Many studies have reported on factors that are probable regulators of A20. A20 interacts with many other proteins to inhibit inflammation, such as A20-binding inhibitor of NF-κB activation 1 (ABIN-1) and Tax1 binding protein 1 (TAX1BP-1). The ABIN family of proteins are negative regulators of NF-κβ and are ubiquitin binders, attaching to the NEMO (NF-kappa-B essential modulator) complex (Wagner et al. 2008). ABIN-1 attaches A20 to the IKK/NEMO complex and aids A20 to exert its deubiquitinating activity (Mauro et al. 2006). ABIN-1 expression depends on NF-κβ. ABIN-1 also inhibits TNF-α mediated apoptosis (Verstrepen et al. 2009). ABIN-1 was found to attach A20 to NEMO/IKKγ in mice and thus facilitates deubiquitination (Mauro et al. 2006). TAX1BP-1 inhibits NF-κβ inflammation (Shembade et al. 2011) and helps A20 to regulate its deubiquitinating activities by recruiting A20 to the polyubiquitin chains to break and interrupt the IKK complex assembly (Verstrepen et al. 2011).

1.3.3 Role of A20 in Inflammatory Bowel Diseases

Many studies have reported the possible role of A20 in gut barrier protection and in control of inflammation. Intestinal homeostasis is markedly affected by the absence of A20, as shown in mouse studies. Mice in which A20 had been knocked out in intestinal epithelial cells showed increased apoptosis, destroyed intestinal epithelium, and invasion of commensal bacteria after treatment with TNF-α (Vereecke et al. 2010). TNF-α is associated with increased epithelial cell shedding and apoptosis in the gut (Bruewer et al. 2003), which definitely disrupts the gut barrier. Mice in which A20 was overexpressed in intestinal epithelial cells, did not have increased intestinal permeability upon LPS administration (Kolodziej et al. 2011), suggesting a possible protective role of A20 against bacteria.

GWAS studies have linked A20 with many human inflammatory conditions, such as IBD, systemic lupus erythematous, and celiac disease (Ma & Malynn 2012). A20 expression was reported to be low in adult CD patients (Arsenescu et al. 2008), along with low response to anti-TNF therapy. Studies profiling A20 in adult IBD patients have shown varying results with regards to disease phenotype and severity. A20 expression has been shown to be low in the colonic and TI mucosa of adult CD patients (Arsenescu et al. 2008), along with low response to anti-TNF therapy. In another study, A20 expression was increased in colonic biopsies of adult UC, but not CD patients (Fernandes et al. 2016). Similarly, A20 expression was found to be decreased in the non-inflamed colon of adult CD patients (Bruno et al. 2015). Thus, there are alterations in the expression of A20 in both adult CD and UC patients; however, the etiology associated

with this change is unknown. Sustained inflammatory regulation is critical for optimizing treatment and hence, identification of factors that regulate inflammation is critical for IBD management. Given the fact that A20 plays a positive role in inhibiting inflammation, and TNF-α-associated intestinal inflammation is a critical factor in IBD pathogenesis, it is necessary to explore the role of A20 in IBD.

1.4 Metabolomics

1.4.1 Current Technology and Challenges

The term 'metabolomics', defined as 'the systemic analysis of the chemical fingerprints of specific cellular processes' was first coined in 1998, when cellular functions of the yeast *Saccharomyces cerevisiae* were described (Oliver et al. 1998). In more practical terms, metabolomics is the study of chemical reactions that involve metabolites. Metabolites are small molecules, the by-products of chemical and enzymatic reactions that occur in the body. A metabolome is defined as the cumulative composition of specific metabolites present and synthesized within a specific eco-system (Fiehn 2002). Over the last decade, research focus has been directed towards small molecule metabolism (SMM), as metabolites and their substrates are involved in various cellular processes, such as protein synthesis and various enzymatic reactions (Hatzimanikatis et al. 2004). The physiological function of any organ is dependent on well orchestrated systematic signalling at the cellular level and involves multifactorial pathways that have integral involvement of genomics, transcriptomics, proteomics, and metabolomics. Every biological process is the result of multiple biological steps, starting from genes encoding

proteins and ending up with various chemicals/metabolites being produced. Thus, 'omics' techniques can help decipher changes at every level, with genomics and transcriptomics indicating the potential for a biological process, proteomics showing the proteins involved in the process, and finally, metabolomics revealing the end result of the biological process. Therefore, focusing on alterations in the metabolome profile may be a helpful tool to gain insight towards the pathological shift in disease process. In 2005, The Human Metabolome Project was founded in Canada with a specific aim to characterize different metabolites and to apply this information to study biomarker and biochemical parameters in various diseases (http://www.hmdb.ca/).

One of the biggest dilemmas challenging the acquisition of gut-related metabolic profiles is the variation in biological samples used for analysis, such as urine, plasma, mucosal biopsies, and stool samples. Due to the diversity in biofluids and tissue samples used for metabolomic analysis, phenotypical correlation of disease with metabolic profile could result in variable results. Another issue is the use of different techniques, such as, nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), that can result in different metabolic profiling (Marchesi et al. 2007; Jansson et al. 2009). Additional factors have to be considered when designating a signature metabolic profile to patients, such as diet, disease stage, and treatment.

1.4.2 Host-microbial Interactions and the Metabolome

The gut is akin to a complex rainforest ecosystem with intertwining interactions between the host, resident microbiota, food, other environmental exposures, and enzymatic reactions that define the boundaries between a healthy and diseased state. Gut microbiota are regulators of immune homeostasis and gut function (Hooper et al. 2012). The gut microbial composition and diversity are largely affected by diet, further emphasizing the importance of host regulation by microbiota (Kau et al. 2011). A life within a life, the gut microbiome have their own functional parameters that have a close relationship with the host metabolic, immune, and signalling pathways (Nicholson et al. 2012). To completely understand this complex relationship, 'omics' methodologies are applied, including, metabolomics, metagenomics, metaproteomics, and metatranscriptomics (van Baarlen et al. 2013). Compared to other omics, metabolomics is able to capture host, microbe, and host-microbe interaction processes, which other omics cannot.

In recent years, metabolomics has been given special attention as a resource to improve our understanding of the mechanisms influencing host-microbial interactions, and to understand the influence of changing dynamics of metabolism on health and disease. Various techniques have been used to assess the metabolome in gut diseases, using NMR (Wissenbach et al. 2016), mass-spectrometry (Ahmed et al. 2016), and liquid chromatography (LC)-MS/MS (Yau et al. 2014).

Most of the studies analyzing metabolomic profiles in IBD have used stool samples. Analysis of stool samples of CD patients revealed an increase in the metabolites related to tyrosine metabolism, such as dopaquinone. Also, increased amounts of 4-hydroxyphenylacetylglycine were present in a subset of colonic CD patients who had higher *Faecalibacterium prausnitzii* abundance, indicating a correlation between metabolites and microbial composition (Jansson et al. 2009). In another study, reduced acetate, methylamine, butyrate, and trimethylamine were found in stool samples of CD and UC patients analyzed with NMR (Marchesi et al. 2007). An additional report describes differences in the stool NMR metabolomic profile of IBD patients compared to the non-IBD group, mainly in short-chain fatty acids related to microbial metabolism, lactate, and amino acids. Notably, leucine, isoleucine, tyrosine, valine, phenylaline, alanine, and glycine were higher in the controls as compared to CD patients, and butyrate was decreased in controls as compared to UC patients (Bjerrum et al. 2015). In fecal samples of UC patients increased cadaverine and taurine levels were detected with NMR (Le Gall et al. 2011).

Therapeutic interventions also impact the composition of metabolites and microbiota in IBD patients. Iron therapy in IBD patients affected the microbial community, with significant decreases in *Collinsella aerofaciens, Ruminococcus bromii*, and *Faecalibacterium prausnitzii.* This change was accompanied by an increase in ceramide phosphate and decrease in methylcytidine and methyl citrate in the control group (Lee et al. 2016).

There is a well-balanced relationship between gut microbiota and metabolites, and a perturbation in this system can lead to inflammation and disease pathogenesis. Butyrate is an important metabolite, produced in the colon as a result of complex carbohydrate fermentation by *F. prausnitzii, Roseburia* spp., *Eubacterium rectale,* etc (den Besten et al. 2013). Butyrate has many beneficial characteristics, including providing an energy source for colonic epithelial cells, decreasing oxidative stress, and enhancing cell differentiation in the colon (Hamer et al. 2008; Furusawa et al. 2013). Succinate, produced through the tricarboxylic acid (TCA) cycle, has recently been described to play an important role in inflammation, by stimulation of dendritic cells by engaging the succinate receptor 1 and by stabilizing the transcription factor hypoxia-inducible factor-1(HIF-1) in macrophages and tumours (Mills & O'Neill 2014). Acetate, produced by fermentation by bacteria, has been shown to be used by *F. prausnitzii* and *Roseburia spp*. to produce butyrate (Duncan et al. 2004).

As the exact etiology of IBD is still unknown, various 'omics' pathways are being currently explored, including a well-developed interest in the gut metabolome. Proteomics and genomics have been extensively studied in order to correlate various genes and proteins with IBD, however, a clear-cut and systematic pattern that leads to defining disease etiology and subsequent prognosis remains unknown. Thus, exploring the metabolome might provide some helpful insight into the pathophysiology.

Chapter 2

Hypothesis and Objectives

2. HYPOTHESIS AND OBJECTIVES

Despite the dramatic advances in science and technology, including availability of GWAS, animal models, and microbiome analyses, major gaps remain in our basic understanding of IBD, especially in children. Capitalizing on the efforts I took in recruiting patients to my first project (focused on identifying epithelial gaps in pediatric IBD), I seized the opportunity to dive into the complexity of IBD and address several critical (and related) factors that could impact disease pathogenesis.

The rationale driving this project was the need to better define the pathogenesis of pediatric IBD through investigating the critical interlinked factors associated with IBD pathogenesis, *i.e.*, the gut barrier, microbial virulence, cytokines, and factors that control inflammation.

I hypothesized that intestinal epithelial gap density in the IBD pediatric population is increased. Barrier disruption in IBD could possibly be due to the effects of bacteria and cytokine-induced inflammation. Thus, along with assessing epithelial gaps, which clearly disrupt barrier integrity, it is important to analyze how bacteria contribute to barrier disruption and the role cytokines, especially TNF-α and the protein A20, play in IBD pathogenesis.

OBJECTIVES:

The general objective of my PhD was to evaluate epithelial gap density in children with IBD and to investigate the potential link between cytokines and microbes in order to delineate their role in disease progression and causation in IBD patients.

The specific aims of my study were to:

Aim 1. Evaluate gap density in pediatric IBD patients, correlate it with changes in cytokines and inflammatory markers, and to establish its prognostic value.

Aim 2. To investigate the role of A20 in IBD pathogenesis.

Aim 3. To study the relationship between microbial virulence, gut environment, and the gut barrier.

Chapter 3

Increased epithelial gap density in the non-inflamed duodenum of children with inflammatory bowel diseases.

3. INCREASED EPITHELIAL GAP DENSITY IN THE NON-INFLAMED DUODENUM OF CHILDREN WITH INFLAMMATORY BOWEL DISEASES.

This manuscript was published in the Journal of Pediatric Gastroenterology and Nutrition (published; December, 2016). The authors are Deenaz Zaidi, Michael Bording-Jorgensen, Hien Q. Huynh, Matthew W. Carroll, Jean-Francois Turcotte, Consolato Sergi, Julia Liu, Eytan Wine.

3.1 ABSTRACT:

OBJECTIVES: Inflammatory bowel diseases (IBD) present commonly in childhood, with yet unknown etiology, but an important role for the epithelial lining is suggested. Epithelial cell extrusion, measured by counting gaps between epithelial cells, is higher in adult Crohn disease (CD) patients than in controls. Our objectives were to compare epithelial gaps in the duodenum of IBD and non-IBD pediatric patients, to study the correlation between epithelial gaps, inflammation, and disease activity, and identify potential mechanisms.

METHODS: Epithelial gap density of the duodenum was evaluated using probe-based confocal laser endomicroscopy (pCLE) in 26 pediatric IBD patients [16 CD, 10 ulcerative colitis (UC)] and 17 non-IBD controls during endoscopy. Epithelial gaps were

correlated with serum inflammatory markers, disease activity indices, and intraepithelial lymphocytes. A panel of 10 inflammatory cytokines and expression of TNFAIP3 (A20; inhibits NF-κβ-induced inflammation) were analyzed in duodenal and ileal biopsies.

RESULTS: Confocal imaging showed significantly higher epithelial gap density in IBD patients, including UC. Interleukin (IL)-2 and IL-8 were higher in duodenal but not ileal biopsies of UC patients. No significant correlation was present between C-reactive protein (CRP), erythrocyte sedimentation rate, disease activity indices, and epithelial gaps in UC patients. In CD patients, CRP positively correlated with epithelial gaps. A20 expression in the duodenum was unchanged amongst non-IBD and IBD cases.

CONCLUSIONS: Epithelial gaps are increased in the duodenum of children with IBD (including UC) but are unrelated to inflammation. This suggests that an altered epithelial barrier is an important systemic feature of pediatric IBD and is not only secondary to inflammation.

Key words: *Intestinal barrier; ulcerative colitis; Crohn disease; cytokines; confocal endomicroscopy*

What is known?

- The epithelial barrier is disrupted in IBD; whether this is a cause or effect of inflammation is unclear
- Increased epithelial cell shedding in the terminal ileum correlates with relapse in adult CD patients
- The duodenum is usually not inflamed in IBD, especially in UC

What is new?

- Epithelial gaps are increased in the non-inflamed duodenum of children with IBD, including UC
- Increased epithelial gaps can occur without inflammation, representing an important structural defect in IBD
- Further mechanistic insight into the role of the epithelial barrier could lead to novel diagnostic and therapeutic applications that will benefit IBD patients

3.2 Introduction:

Inflammatory bowel diseases (IBD), comprising Crohn disease (CD) and ulcerative colitis (UC), are chronic, enervating diseases with long term complications (Zhang & Li 2014) and an unexplained global increase in incidence, especially in children (Abramson et al. 2010; Bernstein et al. 2006; Molodecky et al. 2012; Benchimol et al. 2014).

The intestinal gut barrier is composed of the mucus layer, epithelial cells, and underlying immune cells, (McGuckin et al. 2009) all of which separate the bowel content (and its bacteria) from the immune system and prevent inappropriate immune activation. Epithelial cell shedding can be visualized *in vivo* during endoscopy, at a microscopic level, using probe-based confocal endomicroscopy (pCLE). Confocal endomicroscopy is a successful tool to visualize and study the mechanisms of epithelial cell shedding and gut barrier integrity in mice (Watson et al. 2005). Studies done later also showed the utility of this optical imaging technique for real-time live microscopy of the human gut lining during endoscopy (Liu et al. 2011; Kiesslich et al. 2007; Kiesslich et al. 2012). Epithelial cell shedding is increased in the ileum of adult CD and UC patients, as demonstrated by a higher density of 'epithelial gaps' (spaces left in the epithelium after the extrusion of epithelial cells). Recently, epithelial gaps analyzed with pCLE were found to be higher in the duodenum of adult IBD patients than in healthy controls (Lim et al. 2014a). Moreover, epithelial cell shedding is predictive of prognosis (Liu et al. 2011)

and correlates with disease relapse (Kiesslich et al. 2012) and hospitalization in adult CD patients (Liu et al. 2011).

As compared to CD that can occur anywhere in the gut, UC is limited to the colon and, therefore, disease typically does not occur in the duodenum. Nevertheless, we hypothesized that IBDs (including UC) are systemic bowel conditions where genetic and environmental influences are likely to impact the entire bowel (but only reach the threshold for endoscopic presentation in the colon in UC); this stimulated us to focus on the duodenum, as we expected that more subtle changes (in the absence of inflammation) would better reflect early disease pathogenesis (in contrast to local effects, secondary to inflammation) and support a more global defect in epithelial cell and barrier regulation. Epithelial gaps have not yet been evaluated in children with IBD, compared to controls. As children with IBD present with a more severe phenotype, are more likely to present closer to initial insults, and have less co-morbidities (Ruemmele 2010), investigating potential pathogenic pathways in children could be less confounded by such factors and hence could provide insight into early disease pathogenesis.

3.3 Methods:

Patients: Established and newly diagnosed IBD patients (diagnosed and phenotyped following the Paris Classification) (Levine et al. 2011) and non-IBD controls between the ages of 3-18 years, who were undergoing endoscopic evaluation were asked to consent to the study through the Edmonton Pediatric IBD Clinic (EPIC; 2012-2015) after obtaining approval from the University of Alberta Research Ethics Board (Study ID Pro00023820). We intentionally included both new and established cases of IBD to enable association of findings with variable disease stages and activity levels. The control group included patients undergoing esophagogastroduodenoscopy (EGD) and colonoscopy for indications other than IBD (such as functional abdominal pain, prolonged diarrhea). Patients with macroscopic and histological abnormalities (*e.g.*, celiac disease, gastritis, eosinophilic esophagitis) were excluded, to eliminate effects of upper gut inflammation on the epithelium, as were individuals with known allergy to fluorescein or seafood. Completely healthy children would have been better controls, but they do not require endoscopy. All patients received bowel cleansing with Picosalax® (sodium picosulphate with magnesium citrate), were fasted for at least 6 hours, and were under general anesthesia with propofol for endoscopies, provided by a pediatric anesthetist.

Probe-based confocal laser endomicroscopy (pCLE) and gap quantification: To conduct pCLE, we used the method by Liu *et al.,*(Liu et al. 2011) with modifications. Briefly, epithelial gap density in the duodenum was evaluated using pCLE (Mauna Kea

Technologies, Suwanee, GA, USA) immediately after IV injection of fluorescein (10%, 5 mg/kg weight to a maximum of 250 mg; Alcon, Mississauga, ON, Canada) as a contrast agent. Imaging was conducted in the duodenum for at least 5 minutes, prior to acquisition of biopsies (**Fig. 3.1A**). All images were obtained from several normal-appearing sections of the duodenum and stored as coded video sequences for analysis. Confocal images were later blindly analyzed by two independent reviewers to calculate an epithelial gap density score for each patient. An epithelial gap was defined as a bright white space between epithelial cells (representing fluorescein leakage), which was approximately the same size as adjacent epithelial cells (**Fig. 3.1B**). White spots/lines that did not have measurements similar to nearby epithelial cells were not considered a gap. Images were included only when at least 3/4 of the villus was visible. Three images from each video were selected for analysis. When a gap was observed in a villus, 3 consecutive images were captured to validate its presence. The gap was required to be present in all 3 consecutive images to be defined as one. To quantify gap density, epithelial gaps were counted per villus, normalized to the total number of cells in the villus (Liu et al. 2011). Gap density was calculated as the number of gaps observed per 1,000 epithelial cells, counted only in well-oriented villi (Liu et al. 2011).

Fluorescein quantification in duodenal aspirates: The amount of fluorescein that leaked through the epithelial barrier into the lumen was selected as an additional measure of epithelial permeability. After completing the confocal imaging during endoscopy, 30 ml of 0.9% NaCl were injected into the duodenum through the endoscopy working channel

and the luminal content was aspirated (using a 'suction trap' to collect the fluid) and then frozen at -80° C. Fluorescein concentration in the intestinal aspirate was measured using SpectraMaz M3 microplate reader (Molecular Devices, Sunnyvale, California, USA). Fluorescein stock solution was serially diluted in a 96-well plate with concentrations ranging from 0.00002 to 10 µg/ml and fluorescence readings were obtained to prepare a standard curve. The fluorescein concentration in the duodenal aspirates was quantified in relation to the standard curve**.**

Correlation of disease severity with epithelial gaps: This was conducted by plotting gap density against standardized scoring indices [Pediatric Crohn's Disease Activity Index (PCDAI) (Turner et al. 2012) and Pediatric Ulcerative Colitis Activity Index (PUCAI)] (Turner et al. 2007), and with serum markers of inflammation: erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (Solem et al. 2005).

Cytokine Analysis: Biopsies obtained from the duodenum and TI of patients were snap-frozen at -80 ºC. The biopsies were weighed and a solution composed of 1x PBS, 0.05% Tween-20, protease inhibitor (Sigma P-P8340), 10% BSA concentrate was added to each biopsy at 5 times its weight. The samples were sonicated for 5 seconds followed by centrifuging at 10,000g to pellet cell debris for 10 min at 4 ºC. Supernatants were transferred to new tubes and frozen at -80 ºC for cytokine and protein analysis.

Samples were thawed and cytokine profiles in the duodenal and terminal ileum (TI) biopsies of non-IBD and IBD patients were determined using the V-Plex pro-

inflammatory panel 1 kit (Mesoscale Diagnostics, Rockville, MD, USA), following the manufacturer's instruction, including the following cytokines: Interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and Tumour necrosis factor (TNF)-α, selected because of their involvement in IBD (Neurath 2014b). Cytokines were normalized per gram weight of each biopsy.

Intestinal epithelial lymphocyte quantification: The epithelium of the entire length of the villus was evaluated while avoiding the proximity of small lymphoid aggregates to prevent overestimating the intestinal epithelial lymphocyte (IEL) count. Biopsy sections with the highest number of lymphocytes were chosen to calculate IEL counts, blindly assessed by a pediatric gastrointestinal pathologist (CS). Twenty villi were randomly evaluated from hematoxylin and eosin (H&E) fixed histological sections from each patient and epithelial lymphocytes were quantified per villus. The number of IEL per 100 epithelial cells in five distinct villi, chosen for best orientation, was counted and presented as a mean value. Distribution of the lymphocytes (villous-base, villous-tip, or distribution along the villous-length) was not documented.

Total RNA extraction and TNFAIP3 (A20) reverse transcription PCR: Duodenal biopsies were collected during endoscopy and homogenized in Trizol (Life Technologies, Burlington, ON, Canada). RNA was extracted with Qiagen RNeasy Kit (Qiagen, Mississauga, ON, Canada) and quantified with Nano-Drop ND-1000 Spectrophotometer

(Thermo Fisher Scientific, Wilmington, DE, USA). RNA (500 ng) was reverse transcribed using the Qiagen Quantitect Reverse Transcription kit and cDNA was used for qRT-PCR. qRT-PCR was conducted on 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR cycle parameters were as follows: activation of the polymerase at 50ºC for 2 minutes, initial denaturing of template DNA at 94ºC for 10 minutes, 35 denaturing cycles at 95ºC for 15 seconds, annealing at 58ºC for 15 seconds, and elongation at 72ºC for 30 seconds. *Glyceraldehyde phosphate dehydrogenase* (GAPDH) was used as a housekeeping gene for relative quantification of the transcript amount. Gene expression of *TNF-α-Induced Factor 3* (TNFAIP3; also known as *A20*) was calculated using the $2^{-\Delta\Delta CT}$ method. Specific primers were 5'-CATCACTGCCACTCAGAAGA-3' and 5'- AAGTCACAGGAGACAACCG-3' for GAPDH, and 5'- GAGAGCACAATGGCTGAACA-3' and 5-'TCCAGTGTGTATCGGTGCAT-3' for A20 (Bertiaux-Vandaële et al. 2011; Liu et al. 2013a).

Statistical analysis: Data were analyzed using Graph Pad Prism (Graph Pad Software, San Diego, CA, USA). Comparison between three study groups was done using ANOVA and Mann-Whitney tests. Statistical significance was determined as $P < 0.05$. Correlation analysis was done using Pearson's correlation coefficient.

3.4 Results:

Patients: Complete data are available for a total of 43 patients, including 17 non-IBD controls, 16 CD, and 10 UC patients (10 controls and 5 CD patients were excluded for presence of inflammation anywhere on EGD, and 9 patients were excluded for technical reasons, such as poor video quality or software failure). The median age of patients was similar between groups. Of the CD cohort, 8 patients were newly diagnosed and 8 were previously diagnosed. None of the UC patients were assessed at initial diagnosis. Patient characteristics, including treatments at time of endoscopy, are described in **Table 3.1**.

Table 3.1: Patient Characteristics

Demographic	*Non-IBD (n=17)*	*CD (n=16)*	*UC (n=10)*
Age (mean ± SEM)	12.24 ± 1.46	12.76 ± 1.25	11.83 ± 0.54
Sex (m/f)	12/5	7/9	3/7
New onset	N/A	8	0
Follow-up	N/A	8	10
Treatments (%)			
5-aminosalicylic acid	-	0	50
Sulfasalazine	-	9	10
Prednisone	-	0	40
Azathioprine	-	15	50
Methotrexate	-	16	10
Infliximab	-	15	30

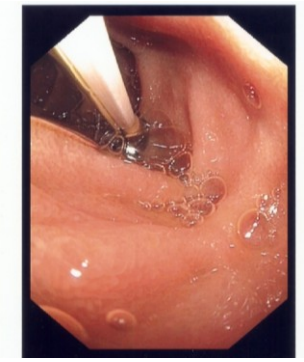
IBD, inflammatory bowel diseases; CD, Crohn disease; UC, ulcerative colitis; f, female; m, male.

Epithelial gap density: We evaluated the duodenum of patients with both active and inactive disease using pCLE to enable assessment of the effects of disease activity on gaps. Epithelial gap density was significantly higher in IBD patients [**Fig. 3.1C**: CD 11.09±3.53 (mean±SEM {standard error of the mean} gaps/1000 epithelial cells); UC: 9.58±4.30; Non-IBD: 0.98±0.67; ANOVA: p=0.04. N = 17 non-IBD, 16 CD, & 10 UC patients].

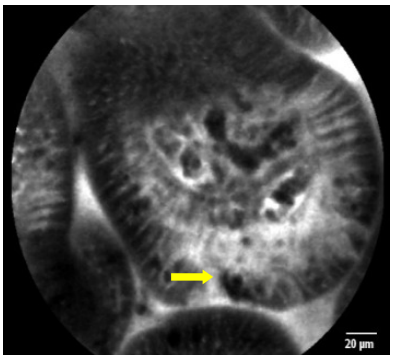
Fluorescein quantification in intestinal aspirates: Fluorescein leaked through epithelial gaps, collected in duodenal aspirates, was significantly higher in IBD than in non-IBD patients (**Fig 3.1D**: CD 3.17±0.8; UC: 5.5±1.3; Non-IBD: 1.4±0.5 μg/ml; Mann-Whitney test: $P<0.05$), validating a barrier defect.

Figure 3.1: Duodenal epithelial gap density is increased in pediatric IBD patients

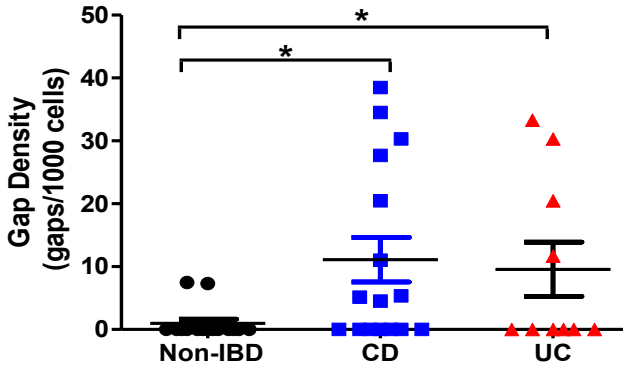
A.



B.



C.



D.

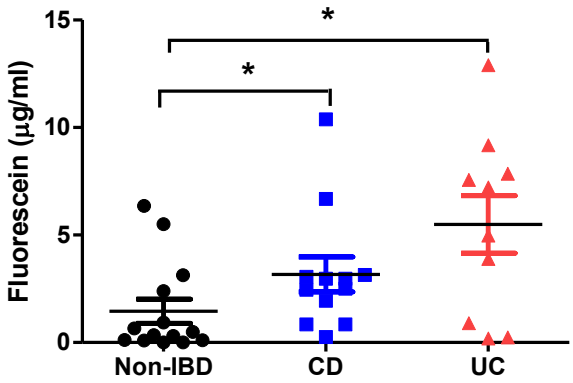


Fig. 3.1

Epithelial Gap Density in the duodenum of IBD and non-IBD patients was analyzed using probe-based confocal laser endomicroscopy (pCLE).

A. pCLE probe obtaining confocal images of the duodenum during esophagogastroduodenoscopy.

B. Representative image of the duodenum obtained using pCLE, showing an epithelial gap (yellow arrow). Fluorescein leakage can be seen as a white color in the gap, spreading into the lumen. Measurement bar: 20μm.

C. Epithelial gap density in IBD patients is significantly higher than in non-IBD controls. CD: 11.09±3.53; UC: 9.58±4.30; Non-IBD: 0.98±0.67; mean±SEM gaps/1000 epithelial cells. ANOVA: p=0.04. N=17 non-IBD, 16 CD, and 10 UC patients.

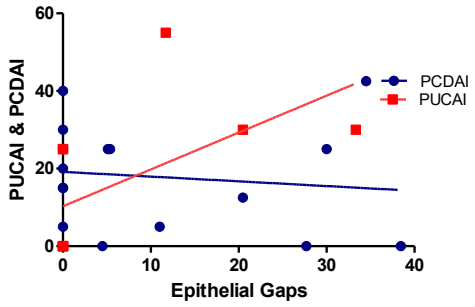
D. Fluorescein leakage into the bowel lumen was significantly higher in IBD patients than in non-IBD patients [CD 3.17±0.8; UC: 5.5±1.3; Non-IBD: 1.4±0.5 μg/ml; Mann-Whitney test, $P<0.05$).

Correlation between epithelial gaps, disease activity, inflammatory markers, and intraepithelial lymphocytes: More epithelial gaps were present in both CD and UC, whether they had active disease or not. There was no correlation between epithelial gaps, PUCAI/PCDAI, and ESR ($P>0.05$, **Fig. 3.2A&B)**. While analysis of the various clinical and inflammatory parameters did not show any correlation with epithelial gaps in UC patients, there was a positive correlation between CRP and epithelial gaps in CD patients ($P<0.01$, **Fig. 3.2C)**. This signifies that epithelial gap density is high in some IBD patients despite normal serum inflammatory markers and low disease activity.

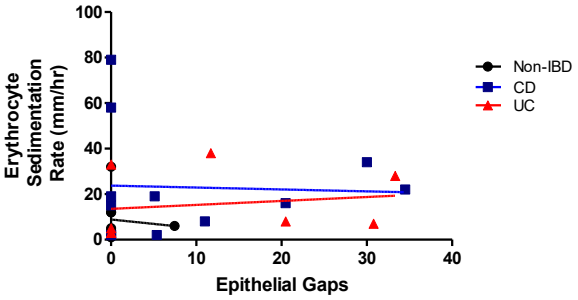
There was no correlation between the intestinal lymphocyte count and epithelial gaps in IBD and non-IBD patients **(Fig. 3.2D)**. This further supports our observation that epithelial gaps are present even in the absence of inflammation.

Figure 3.2: Correlation Between Disease Activity, Inflammatory Markers, Tissue Intraepithelial Lymphocyte Count, and Gap Density

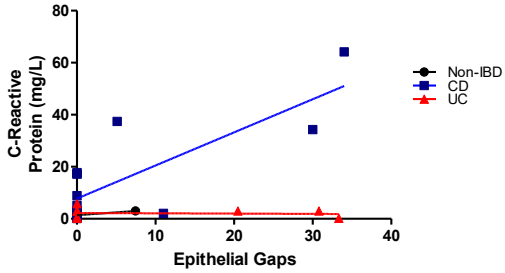
A.



B.



C.



D.

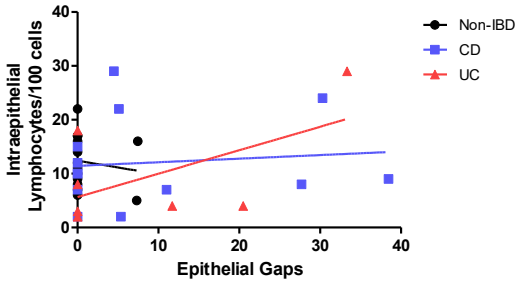


Fig 3.2:

Epithelial gap density is not correlated with disease activity indices [PCDAI and PUCAI (**A**), PCDAI; Pearson r coefficient=0.013, P=0.64. PUCAI; Pearson r coefficient=0.50, P=0.08], and ESR [(**B**), Non-IBD; Pearson r coefficient=0.1, P=0.8. CD; Pearson r coefficient=0.05, P=0.89. UC; Pearson r coefficient=0.16, P=0.69]. Epithelial gap density is positively correlated with CRP in CD, but not UC patients [(**C**), Non-IBD; Pearson r coefficient=0.50, P=0.32. CD Pearson r coefficient=0.8, P<0.001. UC; Pearson r coefficient=0.08, P=0.83]. There was no significant correlation between epithelial gap density and intraepithelial lymphocytes in duodenal biopsies of all three study groups, suggesting that epithelial gaps are an earlier mechanistic feature, preceding the onset of frank pathogenesis [(**D**); Non-IBD Pearson r coefficient= -0.13, P=0.65. CD; Pearson r coefficient=0.10, P=0.7. UC; Pearson r coefficient=0.57, P=0.18].

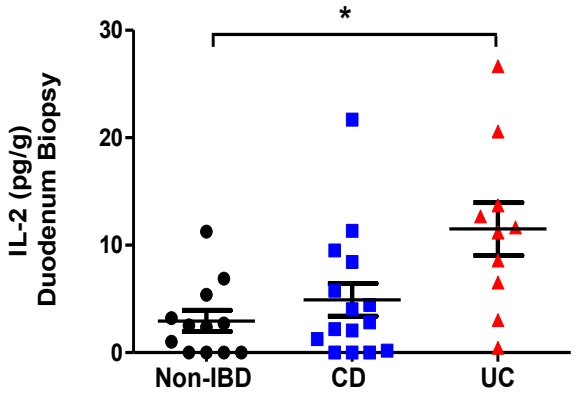
Cytokine levels and A20 expression: IL-2 and IL-8 levels in duodenal biopsies of UC patients were significantly higher than those in non-IBD patients (**Fig. 3.3A&B**; P=0.04 for IL-2 and P=0.02 for IL-8). All other measured cytokines showed no significant difference. There was no difference between duodenal cytokine levels of non-IBD and CD patients. However, TNF-α and IL-8 were significantly higher in the TI of CD patients as compared to tissue from UC and non-IBD patients, confirming that only CD patients indeed had TI inflammation (**Fig 3.3C&D**; $P<0.05$).

A20 (TNFAIP3) expression was unaltered in IBD patients (**Fig. 3.3E**). As TNFAIP3 expression is induced by high TNF-α as negative feedback to inhibit NF-κβ (nuclear factor kappa-light-chain-enhancer of activated B cells), its low expression further indicates an absence of inflammation.

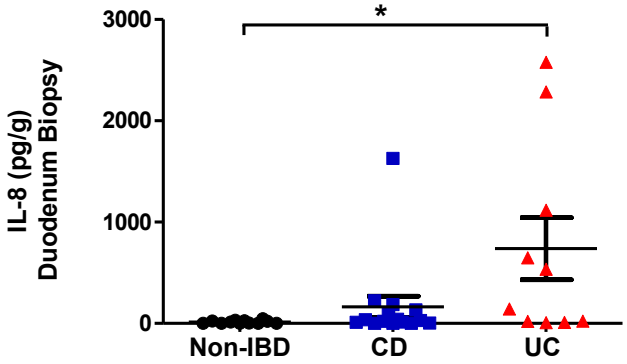
There was no correlation between the duodenum IL-2 and IL-8 of UC patients and gap density (**Fig. 3.3F**).

Figure 3.3: Cytokine and A20 quantification in intestinal tissue biopsies.

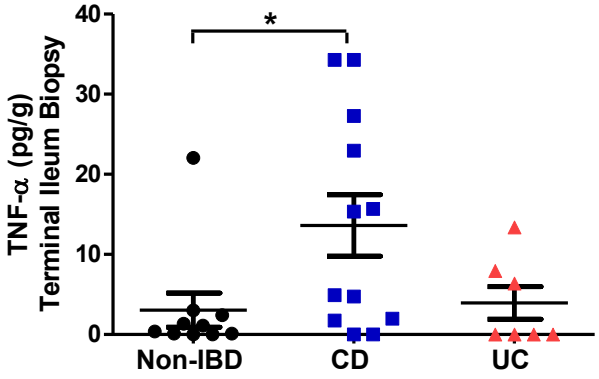
A.



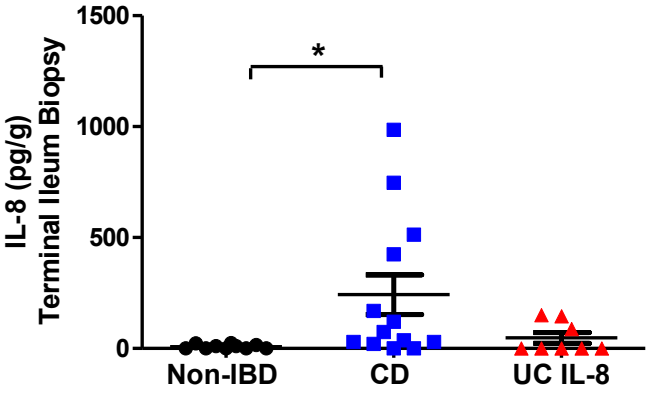
B.



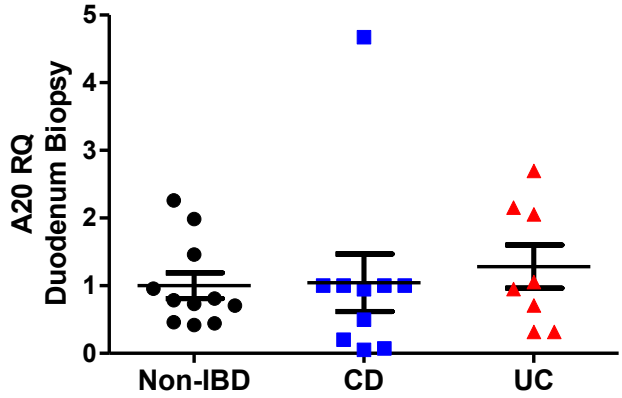
C.



D.


1500
1000
500
0
IL-8 (pg/g)
Terminal Ileum Biopsy
*
Non-IBD
CD
UC IL-8

E.


5
4
3
2
1
0
A20 RQ
Duodenum Biopsy
Non-IBD
CD
UC

F.

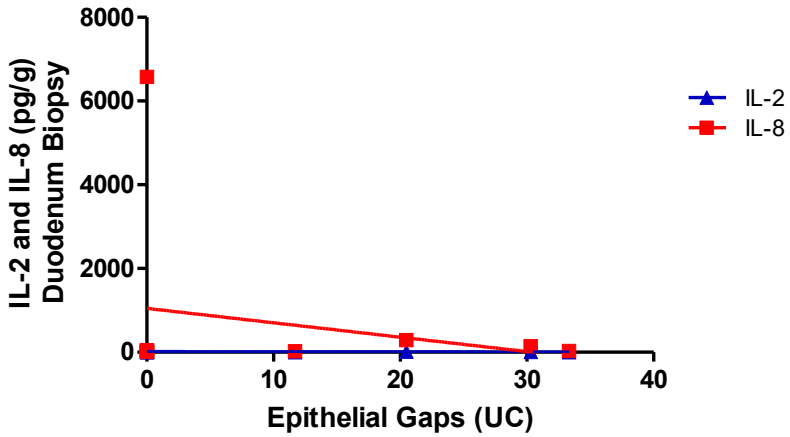

8000
6000
4000
2000
0
IL-2 and IL-8 (pg/g)
Duodenum Biopsy
0
10
20
30
40
Epithelial Gaps (UC)
IL-2
IL-8

Figure 3.3:

Analysis of a panel of 10 cytokines with the Mesoscale V-Plex Multipanel kit showed a significant increase in tissue IL-2 [(**A**) P=0.04] and IL-8 [(**B**) P=0.02] levels in the duodenum of UC patients as compared to non-IBD patients. There was no significant difference between A20 gene expression in IBD and non-IBD patients. TNF-α and IL-8 were significantly higher in the TI of CD patients compared to UC and non-IBD patients (**C&D**; $P<0.05$). (**E**): A20 mRNA relative to GAPDH: Non-IBD; 1.0±0.18, CD; 1.0±0.4, UC; 1.2±0.3, $P>0.05$. There was no correlation between IL-2 and IL-8 levels and gap density in UC patients. [(F) IL-2: Pearson 'r' coefficient = -0.04, P=0.14; IL-8: Pearson 'r' coefficient = -0.22, P = 0.52)].

Taken together, our findings show that epithelial gaps are increased in pediatric IBD patients. The increase in epithelial gaps was independent of the presence of inflammation and was even found in the duodenum of UC patients, indicating that higher cell extrusion and impaired barrier function are significant systemic features of pediatric IBD.

3.5 Discussion:

The complex interplay between the gut barrier, diarrhea, cytokines, and increased bacterial translocation has been designated as a hallmark of IBD (Levine & Wine 2013; John et al. 2011). High resolution imaging of the intestinal epithelial gut, showing that epithelial gaps are present and can be visualized by CLE during ileocolonoscopy in adult patients is credited to Kiesslich and colleagues (Kiesslich et al. 2007). This prompted more work by others and indicated the presence of epithelial gaps to be an important factor in gut barrier function (Liu et al. 2011; Liu et al. 2013). Recently, the presence of epithelial gaps in the duodenum of adult IBD patients has been reported (Lim et al. 2014a).

Cytokines, especially TNF-α, have been shown to be important regulators of gut barrier function (Bruewer et al. 2003). Increased gut permeability is also present in animal models of IL-10 knockouts, (Madsen et al. 1999) as well as in unaffected relatives of CD patients. However, it still remains unclear whether defects in the epithelial barrier represent a fundamental abnormality in IBD or are just the result of inflammation.

Our results show a significant increase in epithelial gaps in the non-inflamed duodenum of UC and CD patients. These findings, especially since they are shown in children, suggest that increased epithelial gaps can occur even in the absence of inflammation, and thus, are an important systemic phenomenon in IBD. Epithelial cell shedding was unrelated to serum inflammatory markers in UC, although it was positively correlated with CRP in CD patients (who also had elevated ileal cytokines). There was no

correlation between epithelial gaps, disease activity indices, and intraepithelial lymphocytes either.

TNFAIP3 (A20) is a NF-κβ-responsive gene involved in negative feedback regulation of NF-κβ activity and also negatively regulates TNF-α-induced apoptosis (Vereecke et al. 2009; Wertz et al. 2015). TNF-α is associated with high epithelial shedding, but TNF-α levels were low in the duodenum of our patients. A few of our patients were on infliximab, which could have lowered potential high levels of TNF-α in the duodenum and suppressed inflammation, but most were not and anti-TNF treatment did not affect the number of gaps. We measured the gene expression of A20, which is usually increased in response to high TNF-α. We found that *A20* gene expression, along with tissue TNF-α level, was low in both new and established patients, further supporting the fact that gaps can occur in the absence of inflammation or high TNF-α in pediatric-onset IBD.

Analysis of tissue cytokines revealed interesting results. While there were no differences in the cytokine levels between non-IBD and CD patients, IL-2 and IL-8 were significantly higher in the duodenum of UC patients. IL-2 influences peripheral survival of T regulatory cells (Tregs) (Barron et al. 2010) and their depletion results in inflammation and triggers autoimmune diseases. IL-2 knockout mice developed colitis similar to human UC (Sadlack et al. 1993). IL-8, a chemokine secreted by macrophages and neutrophils, is present in colonic tissue in IBD patients (Grimm et al. 1996) and is rapidly induced in response to TNF-α or microbes (Hoffmann et al. 2002). This is especially interesting as the duodenum is not typically affected in UC. Thus, both of these

cytokines are related to UC and our findings indicate that early gut barrier disruption in UC can potentially alter immune response even in the absence of frank inflammation. In support of the lack of inflammation in the small bowel of our UC patients, TNF-α and IL-8 were increased in the TI of CD patients but not in UC or non-IBD cases. Further supporting a generalized gut abnormality in UC, we have just shown a reduction in microbial diversity and depleted mucus layer in the non-inflamed ileum of children with UC (Alipour et al. 2015).

We did not assess epithelial gaps in the TI or colon, since we were unable to conduct pCLE in more than one location (to minimize prolongation of procedure and avoid exposure to fluorescein twice). Since the duodenum is less often inflamed than the TI or colon and would, therefore better reflect systemic versus localized changes, we chose to focus on this location.

Our study has several limitations, especially the small sample size. First, recruiting children without gut inflammation for endoscopy is a challenge and we had to remove several IBD patients from our cohort upon finding duodenal inflammation on histology. Not all patients agreed to the prolongation of the procedure and exposure to IV fluorescein. Further, *A20* expression was not available for 6 Non-IBD, 4 CD, and 2 UC patients included in the study. *A20* expression could not be measured in some patients as the basal expression is very low in non-inflamed tissue and hence was undetectable by qPCR in some biopsies. Another limitation is the lack of UC patients evaluated for epithelial gap density at diagnosis due to difficulty in logistics and obtaining consent for the initial diagnostic endoscopy, but our study did include both active and inactive UC.

Beyond better defining disease pathogenesis, future studies on larger cohorts might be able to identify a subset of patients for whom pCLE can be used as a scanning tool to assess the degree of epithelial cell shedding and associated cytokine changes (such as IL-2 and IL-8); whether these changes can be used as markers for disease outcome and treatment response (to anti-TNFs, for example) remains to be determined. In addition, to determine if current medications had any effect on epithelial gaps, follow-up confocal imaging studies will be done on these patients to correlate their treatment regimen with the change in epithelial gap density. *In vivo* confocal microscopy in mice has shown (Marchiando et al. 2012) that after stimulation with TNF-α, tight junction and membrane protein remodeling plays a very important role in maintaining gut barrier integrity. Thus, future studies analyzing tight junction and membrane proteins in biopsies of patients with high gap density would give more information regarding the stability of gut barrier in pediatric IBD.

In conclusion, our study is the first to report the presence of increased intestinal epithelial gaps in children with IBD compared to non-IBD controls. The current data will be followed by a subsequent study to associate gaps with long term disease pattern and consequences, which will provide additional information regarding changes in epithelial barrier and disease progression. This will help elicit if the presence of more gaps is related to increased flares and relapse, and thus, will highlight their possible prognostic value. Increased epithelial gaps in normal-appearing mucosa (validated by histology, endoscopy, and unaltered *A20* expression) suggests that epithelial gaps could be a

physical defect present in pediatric IBD even in the absence of frank inflammation, and in UC, can mediate immune response, which may propagate inflammation elsewhere.

Chapter 4

Prognostic value of probe-based confocal laser endomicroscopy in pediatric inflammatory bowel diseases

4. PROGNOSTIC VALUE OF PROBE-BASED CONFOCAL LASER ENDOMICROSCOPY IN PEDIATRIC INFLAMMATORY BOWEL DISEASES

4.1 Abstract:

Introduction: Inflammatory bowel diseases (IBD), including Crohn disease (CD) and ulcerative colitis (UC), are increasing in children, especially in Canada. We have recently demonstrated increased epithelial gaps in the non-inflamed duodenum in CD and UC patients using probe-based confocal laser endomicroscopy (pCLE), an optical imaging technique that enables visualization of the mucosal surface during endoscopy. Increased epithelial gap density in patients was unrelated to inflammatory parameters or disease activity. The aim of the present study was to determine if increased epithelial gap density could predict the clinical course of IBD patients.

Methods: A total of 26 IBD patients (16 CD and 10 non-IBD cases) were followed from the time of initial pCLE imaging for a minimum of 12 months. Clinical outcomes analysed included the occurrence of significant clinical events (hospitalizations, surgeries, disease flares), alterations in serum inflammatory parameters (CRP, ESR), and changes in disease activity indices. Kaplan-Meier plots were used to analyse event-free survival probabilities and log-rank tests were performed to determine the differences between the groups. Cox proportional hazard models were used to determine if gap density was a predictor of risk of clinical events or inflammatory parameters. Student's *t*-

test was used to compare inflammatory parameters and Spearman's correlation tests were used to determine correlation of gap densities with clinical outcomes.

Results: Patients were followed for a minimum of 12 months. Only two patients developed clinical flares in the follow up period. While the presence of high epithelial gaps did not predict the risk of clinical events or alterations in inflammatory parameters (CRP, ESR) in IBD patients, CD patients with high epithelial gap density who were treated with infliximab were found to have a significant reduction in ESR levels at the 12-month follow up time point.

Conclusion: The study shows for the first time, that infliximab treatment significantly reduces ESR levels in pediatric CD patients with high epithelial gap density. Infliximab treatment did reduce ESR levels in patients with normal gaps too, however this effect was more marked in patients with high gaps. Future studies evaluating epithelial gap density in pediatric IBD patients and its relation with clinical parameters and infliximab might be helpful in patient selection and defining better treatment options.

4.2 Introduction:

Inflammatory bowel diseases (IBD), including both Crohn disease (CD) and ulcerative colitis (UC) are common, debilitating, intestinal disorders that frequently present in childhood (Benchimol et al. 2014). The causes of IBD remain unknown; theories support a model where unremitting inflammation, likely triggered by environmental exposures, leads to intestinal damage in genetically susceptible hosts (Atreya et al. 2008). This is accompanied by gut barrier dysfunction, although it remains unclear whether this is the cause or result of intestinal inflammation. The gut barrier is comprised of a single layer of epithelial cells, enhanced by a mucus layer, antimicrobial proteins, IgA, and resident bacteria, which all separate the luminal bacteria from the submucosal immune system. Epithelial cells originate from stem cells in the crypt base and migrate to the villi (in the small bowel) until they are shed from the villus tip into the lumen. Increased intestinal epithelial cell shedding has been reported as a feature of IBD (Liu et al. 2011; Watson et al. 2009) and has been shown to be predictive of disease relapse and hospitalization in adult patients (Turcotte et al. 2012).

Probe-based confocal laser endomicroscopy (pCLE) enables live imaging of the intestinal mucosa during endoscopy and allows visualization of epithelial cell gaps *in vivo* (Kiesslich et al. 2012; Liu et al. 2011). Interestingly, in IBD, increased epithelial cell shedding has not only been limited to the colon and terminal ileum; rather, two independent studies have shown the phenomenon in the duodenum, a usually unaffected site in IBD patients, especially UC. Lim *et al*., (Lim et al. 2014b) documented increased

epithelial gaps in the duodenum of adult IBD patients. We also found increased epithelial gap density, defined as the number of epithelial gaps per 1000 epithelial cells, in the duodenum of both CD and UC patients (Zaidi D et al. 2016). Epithelial gaps were unrelated to local inflammation or disease activity in our study, suggesting that these gaps could represent a baseline defect in the gut barrier in pediatric IBD patients that is not secondary to inflammation.

Further probing the outcomes of this significant feature in IBD patients, we wanted to analyze if the presence of increased epithelial gap density could predict disease course and/or response to therapy. Thus, we conducted a follow up of the patients enrolled in our previous study (Zaidi D et al. 2016) in order to determine the clinical course of patients who had increased epithelial gaps in the duodenum, as measured using pCLE. Our objective was to analyze if patients with higher epithelial gaps had increased incidence of clinical complications (described as IBD-related hospitalization, surgeries, or flares), changes in disease activity indices, or altered inflammatory markers (CRP and ESR) over the period of 12-24 months. In a subanalysis, we also aimed to see if gaps could predict response to therapy.

Our findings demonstrate that increased epithelial gap density did not predict the risk of clinical events, alterations in inflammatory parameters (CRP, ESR), or disease activity scores in IBD patients. However, CD patients with high epithelial gap density who were treated with infliximab did demonstrate a significant reduction in ESR levels at the 12 month follow up time point, as compared to those with normal gaps treated with the medication. Thus, it is possible that the presence of high epithelial gaps, as

determined by pCLE, could potentially be used as a marker to select infliximab treatment, amongst other clinical parameters.

4.3 Methods:

Patients: Patients included in the study were both established and newly diagnosed IBD patients (3-18 years of age), who had epithelial gaps quantified in the duodenum using pCLE during esophagogastroduodenoscopy conducted as part of our previous study (Zaidi D et al. 2016). A total of 26 IBD patients were included in the study, including 16 CD, and 10 UC patients. All patients included had consented to participate in both the confocal study and subsequent follow up, as approved by the University of Alberta Research Ethics Board (Study ID Pro00023820). The initial study was conducted from 2012-2015, and the follow up ranged between 12-24 months.

Clinical outcomes: The clinical follow up of the patients included documentation of significant clinical complications (described as hospitalization, surgeries, or disease flares), serum inflammatory parameters [(erythrocyte sedimentation rate (ESR), C-reactive protein (CRP)], (Solem et al. 2005), and use of medications. All data were obtained through routine clinic visits at the Edmonton Pediatric IBD Clinic (EPIC) and extracted from Alberta Netcare and eClinician, provincial electronic medical records managed by Alberta Health Services, which store patients' complete medical records, including hospitalizations, medications, and laboratory investigations. Flares were defined as Pediatric Crohn's Disease Activity Index (PCDAI) (Turner et al. 2012) or Pediatric Ulcerative Colitis Activity Index (PUCAI) (Turner et al. 2007) scores of > 10.

Statistical Analysis: The cut-off point for normal gap density was determined based on a similar previous study (Turcotte et al. 2012). Epithelial gap densities of > 6% (the estimated 94th percentile of the non-IBD group) were considered to be abnormal. Kaplan-Meier plots were used to determine event-free survival probabilities, with log-rank tests to determine the differences between study groups. Cox proportional hazard models were used to determine if gap density was a predictor of risk of clinical events or inflammatory parameters. Comparison between the inflammatory/disease activity parameters (ESR, CRP, PCDAI, and PUCAI) were done with Student's *t*-tests. Correlation between epithelial gap density, clinical events, and inflammatory parameters was done using Spearman's test. The differences in inflammatory markers of patients on various treatments were analyzed with Student's *t*-test. The statistical analysis was done with Graph Pad (San Diego, CA, USA) and XLStat (Newyork, NY, USA). A *P* value of <0.05 was considered to be significant.

4.4 Results:

Patients Characteristics: Of the CD cohort, 8 patients were newly diagnosed and 8 were previously diagnosed and on active therapy at the time of epithelial gap calculation. The UC cohort included only patients with a previous diagnosis. Of the CD group, 10 patients were determined to have normal gap density, whereas 6 had high gap density. In the UC group, 6 patients had normal and 4 had high epithelial gaps in the duodenum. In the CD normal epithelial gap group, 4 patients were followed for at least 18 months, and 6 for 24 months; whereas, in the CD high gap group 3 patients were followed for 18 and 3 for 24 months. In the UC group, 7 patients were followed for 18 months and 3 for 24 months.

Proportion of IBD patients with normal inflammatory parameters in the follow-up period varied between CD and UC patients: Analysis of clinical outcomes was conducted at 12 months post confocal imaging. The 12-month time-point was selected as there was variation in the follow up time periods amongst patients, and clinical data was available for all patients at 12 months.

At 12 months after epithelial gaps were analyzed, the percent of patients with normal clinical parameters (PCDAI/PUCAI <10, lab values of CRP <8 mg/L, and ESR <15 mm/hr) was 70 and 84% in CD patients with normal and high epithelial gaps, respectively, and 100 and 75% in UC patients with normal and high epithelial gaps, as shown in **Fig. 4.1**.

Figure 4.1: Percent of normal inflammatory parameters at 12 month follow up does not differ in IBD patients with different epithelial gap densities.

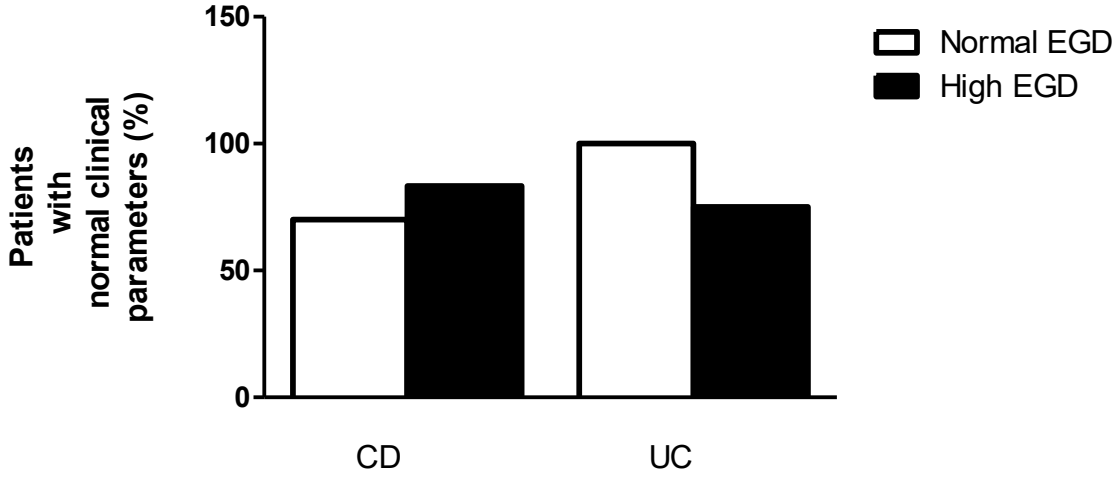


Figure 4.1:

The percentage of patients with normal clinical parameters (PCDAI/PUCAI <10 AND normal lab values of CRP and ESR) varied between IBD patients, with 70 and 84% in CD patients with normal and high epithelial gap density, respectively, and 100 and 75% in UC patients with normal and high epithelial gap density, respectively.

Event-free survival probabilities and inflammatory parameters did not differ significantly between patients with normal and high epithelial gap density: Analysis of event-free survival probabilities was done with the Kaplan-Meier method and comparison was made with log-rank tests. In all instances, there was not a significant difference between patients with normal and high epithelial gap density. There was only one incident of hospitalization in a patient at the 12-month point in the CD group with normal epithelial gaps and one incident of flare in one patient in each group at the 12-month mark. Two CD patients with normal epithelial gaps had elevated CRP levels at 24 months. At 12 months, 2 patients with high and 3 patients with normal epithelial gaps in the CD group had increased ESR, and at 24 months 1 patient with high epithelial gaps had elevated ESR.

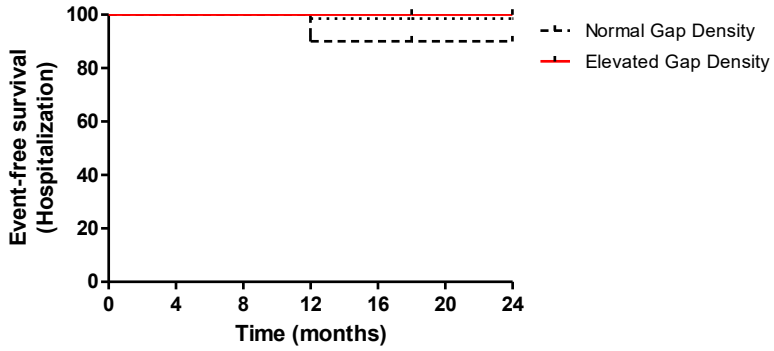
In the UC group there were no significant clinical events or abnormal clinical parameters, except in one patient of the high epithelial gap group who had high CRP levels at the 12 month mark.

Although patients in the normal and high epithelial gap groups had different trends of clinical events/inflammatory markers, there was no significant difference in any inflammatory parameter or events between the groups, (**Fig, 4.2A-E**: log-rank and Gehan-Breslow-Wilcoxin tests $P>0.05$).

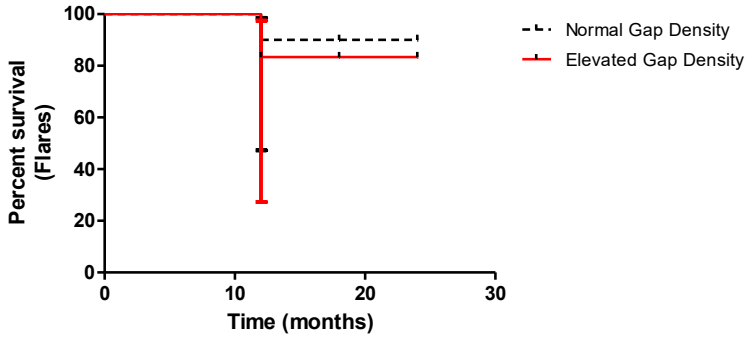
Cox proportional hazard models did not identify gap density to be a predictor of clinical events or inflammatory markers (CRP, ESR, disease activity indices).

Fig 4.2. Incidence of clinical events and increased inflammatory parameters is not significantly different in CD patients with high epithelial gaps

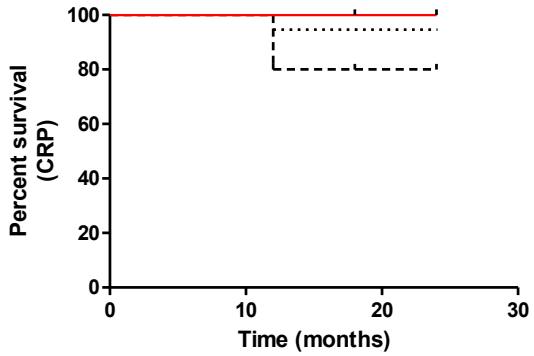
A.



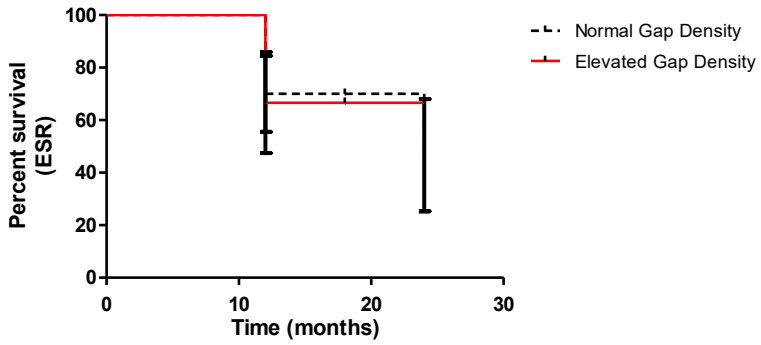
B.



C.



D.


Percent survival (ESR)
100
80
60
40
20
0
0
10
20
30
Time (months)
Normal Gap Density
Elevated Gap Density

E.

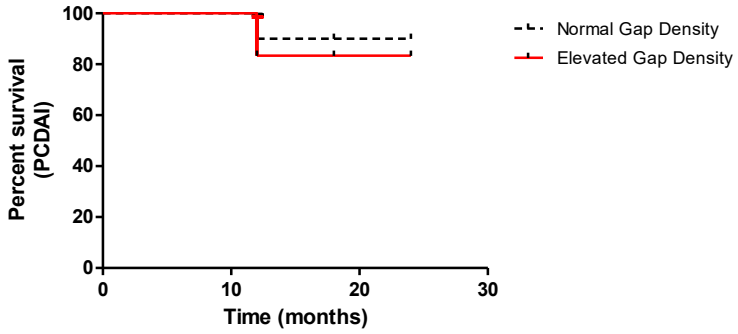

Percent survival (PCDAI)
100
80
60
40
20
0
0
10
20
30
Time (months)
Normal Gap Density
Elevated Gap Density

Fig 4.2:

Analysis of event-free survival probabilities and inflammatory parameters did not show a significant difference between patients with normal and high epithelial gap density. **A**. Hospitalization, **B**. Flares. **C**. CRP, **D**. ESR, **E**. PCDAI. Kaplan-Meier method, log rank test, $P>0.05$.

Elevated epithelial gap density predicts a reduction in ESR in CD patients treated with infliximab: In order to further explore why the incidence of clinical events varied amongst CD patients, we looked at their treatment regimen. In the CD group, patients with higher gap densities when treated with infliximab had a significant decrease in ESR levels from baseline to 12 months, (**Fig. 4.3**, Student's *t*-test, $P<0.05$). While the ESR levels of patients with normal gap density were also reduced with infliximab treatment, this effect was not as significantly marked as that observed in patients with higher gaps. The reduction in the ESR levels of patients with normal gap density from baseline to 12 months was not significant. In the CD group with normal epithelial gaps, 5 were treated with infliximab, and had normal CRP, ESR, and PCDAI levels during the follow up period. Amongst CD patients who had high epithelial gaps, 3 patients were on infliximab. There was no correlation between epithelial gap density, CRP, and ESR in any group, whether they were treated with infliximab or not.

Figure 4.3: **CD patients with elevated epithelial gap density at baseline who were treated with infliximab displayed a reduction in ESR after 12 month therapy**

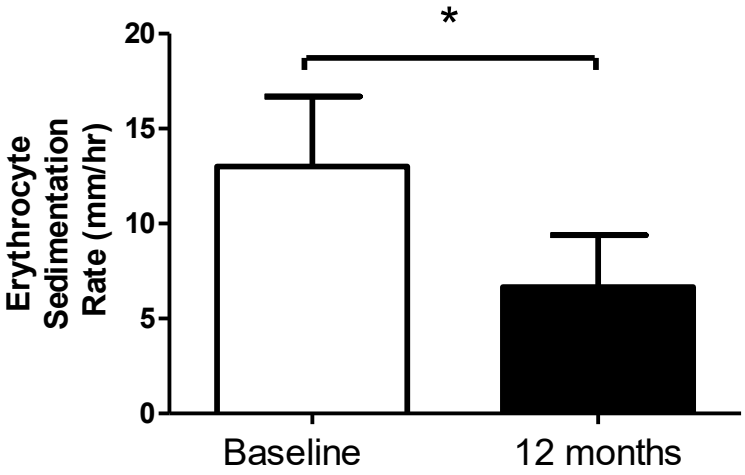


***Figure 4.3**:*

ESR levels of CD patients who had elevated gap density and were treated with infliximab were significantly decreased at the 12-month follow up point. Student's *t*-test, $P<0.05$.Baseline ESR: 13 ± 3.6 mm/hr; 12-month ESR: 6.6 ± 2.7 mm/hr (mean ± SEM).

4.5 Discussion:

This study comprised the clinical follow up of IBD patients enrolled in our previous study. We had previously found that epithelial gap density was increased in the duodenum of both CD and UC patients and was unrelated to inflammation. The aim of our study was to determine if the presence of higher EGD correlates with and predicts clinical events (hospitalizations, flares), inflammatory parameters (CRP, ESR), disease activity indices (PCDAI/PUCAI), and response to treatments.

We did observe a variation in patients who had maintained normal clinical parameters (PCDAI/PUCAI <10, normal lab values of CRP and ESR) within the follow up period. The percent of IBD patients who achieved normal scores was higher, but not statistically significant in the CD group with high epithelial gap density; this suggests that epithelial gaps could serve as predictors of response, similar to CRP, which, when elevated prior to initiation of treatment predicts response to infliximab (Jurgens et. al 2011). There seems to be an opposite effect in the UC group, although, only one patient with high epithelial gap density had a high CRP level at 12 months.

Analysis of clinical events and inflammatory parameters in the follow up period showed that there was neither a specific trend, nor a significant difference between patients with normal and high EGD. While this did not support our hypothesis, we cannot exclude that a lack of association is only due to the very low rate of flares/complications/active disease seen in our cohort during the follow-up period. We next looked at the correlation of epithelial gap density with CRP, ESR, PCDAI, and

PUCAI and did not find any significant correlation. However, at the 12 month follow up point, we did find a significant decrease in serum ESR levels in CD patients with higher epithelial gap density treated with infliximab.

TNF-α plays a significant role in gut barrier disruption (Bruewer et al. 2003). Infliximab is successfully used to achieve remission in many IBD patients (Guo et al. 2013). However, there are certain adverse effects associated with infliximab, such as increased risk of infections (Veereman-Wauters et al. 2012) and lymphomas (Kelsen et al. 2011), highlighting the need to select patients for infliximab therapy through detecting predictors. Thus, if our findings are confirmed in a larger cohort, identification of high epithelial gap density with pCLE could potentially be used as an aid to deciding to initiate infliximab therapy, as it is associated with a significant drop in ESR levels, and could possibly be a reason for lesser clinical events observed in the CD patients with high epithelial gap density. The small sample size of the study was a limitation. Clearly, additional larger cohorts are needed to confirm the clinical importance of pCLE to determine if increased epithelial gap density in CD patients is indeed an indicator of response to infliximab therapy in order to gain remission. Increased epithelial gaps could signify a barrier defect that anti-TNF therapy could correct.

We have shown, for the first time, that CD patients with high epithelial gap density, as determined by imaging the non-inflamed duodenum in CD patients, who are then treated with infliximab, tend to have significantly lower ESR levels in the follow-up period. This finding suggests that pCLE could serve as a marker to predict response to therapy; similar observations have been for CRP, which when elevated prior to treatment,

predicts response to infliximab (Jurgens et. al 2011). Thus, pCLE has a potential for being a marker of biological processes that could help target and direct drug therapy in pediatric CD patients; larger studies are required to further validate and explore this option.

Chapter 5

Quantitative analysis of capillary flow rate in the duodenum of pediatric inflammatory bowel disease patients.

5: QUANTITATIVE ANALYSIS OF CAPILLARY FLOW RATE IN THE DUODENUM OF PEDIATRIC INFLAMMATORY BOWEL DISEASE PATIENTS.

This manuscript has been accepted for publication in the Journal of Pediatric Gastroenterology and Nutrition. The authors are Deenaz Zaidi, Lucas Churchill, Hien Q. Huynh, Matthew W. Carroll, Rabin Persad, Eytan Wine.

5.1 ABSTRACT:

Background: Inflammatory bowel diseases (IBD), including Crohn disease (CD) and ulcerative colitis (UC), are chronic pediatric diseases. Changes in vasculature are described in IBD but these could be secondary to inflammation and the role in pathogenesis is poorly understood. Assessing circulatory changes in typically unaffected sites in IBD (*e.g.*, duodenum), when inflammation is absent, can identify vascular changes associated with pathogenesis.

Aim: To measure capillary flow rates in duodenal mucosa using probe-based confocal laser endomicroscopy (pCLE) during endoscopy in children with IBD.

Methods: Images of villi with visible blood vessels obtained using pCLE were captured as video sequences. Capillary flow rate (mm/s) was calculated by dividing the distance travelled by blood cells by the duration of the sequence. Flow rates were correlated with various clinical parameters.

Results: Forty-five patients (22 non-IBD, 14 CD, 9 UC) were included in the study. Duodenal capillary flow rates were significantly higher in UC patients (0.75±0.07 mm/s) as compared to non-IBD (0.57±0.03) and CD patients (0.65±0.04). There was no correlation between serum hemoglobin and albumin, disease activity indices, serum inflammatory markers, and capillary flow rates in patients.

Conclusions: This pilot study shows, for the first time, increased capillary blood flow in the duodenum of UC patients that was unrelated to inflammatory markers or disease activity. Thus, early vascular changes can be assessed using pCLE during endoscopy.

Key words: Capillary flow rate, ulcerative colitis, Crohn disease, duodenum, confocal laser endomicroscopy

5.2 Introduction:

Inflammatory bowel diseases (IBD), including both Crohn disease (CD) and ulcerative colitis (UC), are major pediatric health concerns (Burisch & Munkholm 2013). IBD mainly involves the gastrointestinal tract; however, these diseases can also manifest outside the gut, indicative of potentially broad-spread systemic involvement (Patil & Cross 2013; Dotson et al. 2010). While disease extent is typically limited to the colon in UC (in contrast to CD, where the entire bowel can be involved), extracolonic involvement in UC, such as focal gastritis, has also been reported (Lin et al. 2010; Tobin et al. 2001), suggesting that the pathogenesis may reach beyond the colon. We have recently demonstrated increased epithelial gaps (indicative of epithelial cell shedding) in the non-inflamed duodenum of UC patients using probe-based confocal laser endomicroscopy (pCLE), an optical imaging technique that enables live visualization of the mucosal surface (Zaidi D, 2016). Thus, pCLE is a technique that can assist in visualizing changes associated with IBD *in vivo* during endoscopy, and might help in better understanding disease pathogenesis. Mucosal barrier and microbial changes prevail in the non-inflamed terminal ileum of pediatric UC patients (Alipour et al. 2015). These findings indicate that pathological changes can occur outside of colon in UC, despite lack of endoscopic and microscopic inflammation.

Vascular changes have been described in IBD, although it is unclear how they relate to disease pathogenesis (Cromer et al. 2011). A major challenge in studying the pathogenesis of IBD is that many features, including increased vascular flow, could be

the result of inflammation, and not the cause. Increased epithelial gaps in the non-inflamed duodenum of pediatric UC patients indicate that a sub-clinical pathophysiologic process occurs in UC outside the colon (Zaidi D et al., 2016); this could help identify factors that might potentially cause preliminary tissue damage, unrelated to or preceding inflammation. Thus, assessing circulatory changes in the duodenum in the absence of inflammation could better define the early stages of vascular pathogenesis in IBD.

We hypothesized that children with IBD would have alterations in the microvascular flow. Microvascular changes, such as tortuous vessels and fluorescein leakage into the lamina propria, have been assessed with pCLE and found to be abnormal, even in the absence of frank lesions in UC patients, which indicates that pCLE can be used as a diagnostic tool for disease-associated early vascular changes (Buda et al. 2014). pCLE has been used for studying vascular changes in sepsis in porcine models and in duodenum of patients with severe sepsis to quantify capillary length and functional capillary density (Schmidt et al. 2013). However, vascular flow was not assessed in either of these studies. We are the first group to report vascular flow changes in IBD patients, which is indeed a novel application of pCLE for understanding IBD pathogenesis. The objective of the study was to assess microvascular flow in the non-inflamed tissues of IBD patients. High resolution of mucosal circulation and single blood cell tracking can be assessed with pCLE, and enabled us to measure capillary flow rates during endoscopy. By measuring capillary flow rates in duodenal images obtained using pCLE during endoscopy, we show that pediatric UC patients have increased duodenal capillary flow, which was not related to disease activity, inflammatory markers, or hemoglobin levels.

5.3 Methods:

Patients: IBD patients and non-IBD controls (4-17 years old) undergoing endoscopic evaluation were included. Ethics approval (University of Alberta Research Ethics Board Study-ID Pro00023820) was obtained. The non-IBD arm of the cohort included patients undergoing esophagogastroduodenoscopy (EGD) to investigate gastrointestinal symptoms (*e.g.*, abdominal pain, diarrhea), but they were excluded if any mucosal abnormality was found. Patients with any abnormality in the upper GI tract (endoscopic or histologic) or other mucosal lesions were excluded. A major challenge that we faced was difficulty in recruitment, as confocal imaging prolonged the endoscopy procedure and required IV fluorescein. Also, an ideal non-IBD cohort would have included completely healthy children but they do not undergo endoscopy. All patients received Picosalax® (sodium picosulphate with magnesium citrate) for colonoscopy bowel prep. Endoscopies were performed under general anesthesia with propofol.

pCLE and capillary flow rate analysis: Duodenal vascular imaging of patients was conducted using pCLE during endoscopy. Mucosal imaging with pCLE was performed before extracting biopsies in all cases and shortly after initiating anesthesia (upper endoscopy always preceded colonoscopy) in order to reduce variation in anesthesia and procedure time that could affect outcomes. After injection of IV fluorescein (10%, 5 mg/kg, maximum of 250 mg; Alcon, Mississauga, ON, Canada) as contrast media, the confocal probe was inserted through the endoscopy working channel. Mucosal images

were collected by positioning the probe directly against the mucosa **(Fig. 5.1A).** Images were obtained, anonymously stored, and analyzed, using the Cellvizio viewer software (Muana Kea Technologies Inc., Suwanee, GA, USA), separately by two reviewers who were blinded to the study groups.

Images were selected where at least a 1/4 of the villus was visible. Video sequences in which the blood cells were present in a single file were selected for capillary flow rate analysis since more than one parallel file would likely represent larger blood vessels with higher flow rates. Blood vessels with a diameter greater than 20 μm were excluded for the same reason. Blood cells needed to be visible over at least 3 consecutive frames in the video sequence to be included in the analysis. A minimum of 3 individual blood cells were tracked per patient (more when possible). The average of the distances travelled by blood cells were used to determine flow rates for each patient. Distance travelled by blood cells was measured using an epithelial cell as an anatomical reference point with the Cellvizio software diameter tool. The distance travelled was divided by sequence duration to calculate the capillary flow rate (μm/ms or mm/s; **Fig. 5.1B**).

Confocal endomicroscopy prolonged the endoscopy procedure by 5-7 minutes. Fluorescein is commonly used in ophthalmology practice and it has been safely used during confocal endomicroscopy procedures in pediatric patients (Venkatesh et al. 2009). Some reported adverse reactions to intravenous injection of fluorescein include rash, hypotension, nausea, and vomiting, but these occur rarely (Kwiterovich et al. 1991). The risk of severe side effects, such as bronchospasm and cardiac arrest, is extremely low (0.05%) (Yannuzzi et al. 1986).

Correlation of capillary flow with disease activity: The correlation between capillary flow and disease features was conducted by plotting capillary flow rates against serum inflammatory markers [erythrocyte sedimentation rate (ESR), C-reactive protein (CRP)] (Solem et al. 2005) and against the standardized UC scoring index, Pediatric Ulcerative Colitis Activity Index (PUCAI) and Pediatric Crohn Disease Activity Index (PCDAI) (Turner et al. 2007; Turner et al. 2012). Serum hemoglobin and albumin levels were also compared to flow rates.

Cytokine Analysis: As described in our recently published study (Zaidi D et al. 2016; Chapter 3), cytokine profiles in the duodenal biopsies of all non-IBD and IBD patients were analyzed using the V-Plex pro-inflammatory panel 1 kit (Mesoscale Diagnostics, Rockville, MD, USA) and included the following cytokines: Interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and tumour necrosis factor (TNF).

Statistical analysis: Data were analyzed using Graph Pad Prism (San Diego, CA, USA). Comparison between study groups was done using ANOVA and correlations were conducted using Pearson's correlation coefficient. Interobserver and intraobserver variability was determined by Cohen's kappa (κ) coefficient. Statistical significance was determined as $P < 0.05$.

5.4 Results:

Patients: Confocal images were obtained from 57 patients, of which 12 were excluded as they did not meet the inclusion criterion (low image resolution; tissue inflammation). Of the 12 patients who were excluded 3 IBD and 5 non-IBD had poor resolution of images. All 4 patients who were excluded for evidence of inflammation in the duodenum were non-IBD patients. Complete data were available for 45 patients, including 22 non-IBD controls, 14 CD, and 9 UC patients. Of the CD cohort, 8 were newly diagnosed and 6 were previously diagnosed. All UC patients were previously established cases. Patient characteristics are described in **Table 5.1**.

Table 5.1: Patient Characteristics

Demographic	***Non-IBD (n=22)***	***UC (n=9)***	***CD (n=14)***	***'P' value***
Sex (m/f)	12/10	3/6	10/4	
New onset	N/A	0	8	
Follow-up	N/A	9	6	
Age (mean ± SEM)	11.5± 0.8	11.83 ± 0.54	12.44 ± 0.88	0.29
Hemoglobin (g/L)	133.8 ± 2.1	123.9 ± 5.8	124.3 ± 4.4	0.09
Albumin (g/L)	44.53 ± 0.6	43.38 ± 0.6	40.75 ± 1.5	0.25
Treatments (%) at time of endoscopy				
5-aminosalicylic acid	-	44	0	
Sulfasalazine	-	11	7	
Prednisone	-	33	0	
Azathioprine	-	44	28	
Methotrexate	-	11	14	
Infliximab	-	23	14	

IBD, inflammatory bowel diseases; CD, Crohn disease; UC, ulcerative colitis; f, female; m, male.

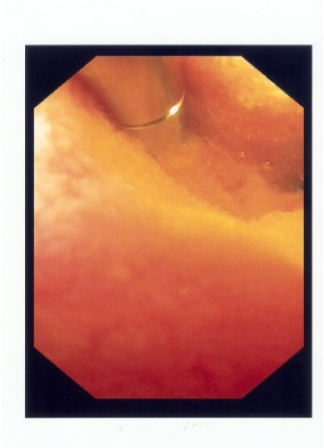
Capillary flow rates: Confocal images were analyzed to quantify capillary vascular flow (example shown in **Fig. 5.1B**). A minimum of 3 distance points travelled by a blood cell was recorded per patient (more where possible). Data sets from both observers were analyzed and the average measurements reported. The number of capillary flow measurements recorded per patient were: non-IBD: 4.4 ± 0.25, CD: 3.90 ± 0.3, UC: 4.2 ± 0.4.

Duodenal capillary flow rates were significantly higher in UC patients [Fig. **5.1C**; non-IBD controls: 0.57±0.03; UC: 0.75±0.02; CD: 0.65±0.02 (mean±SEM; capillary flow rate mm/s); ANOVA, $P<0.05$, N=22 Non-IBD, 14 CD, and 9 UC patients]. Cohen's κ coefficient interobserver variability was 0.82, suggesting strong agreement between the blinded observers. Mean values of capillary flow rate measurements by reviewer 1 were: non-IBD: 0.55 ± 0.04, UC: 0.74 ± 0.06, CD: 0.63 ± 0.04, and those of the second reviewer were non-IBD: 0.59 ± 0.02, UC: 0.8 ± 0.05, CD: 0.67 ± 0.04 mm/s. Intraobserver reliability of the measurements was determined by a second blinded analyses by reviewer 1, and showed a very high degree of correlation between the first and second sets of readings (Cohen's κ coefficient was 0.98).

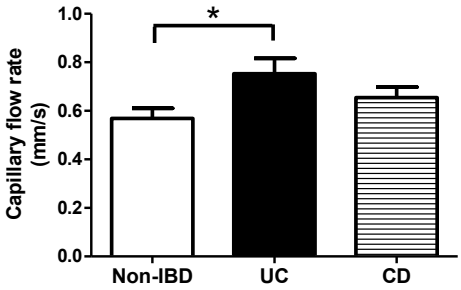
None of the included patients had duodenal inflammation on endoscopy or histology; this suggests that increased vascular flow occurs in these patients in the absence of frank or microscopic lesions.

Figure 5.1: Duodenal Capillary Flow is Increased in Pediatric UC Patients

A.



C.



B.

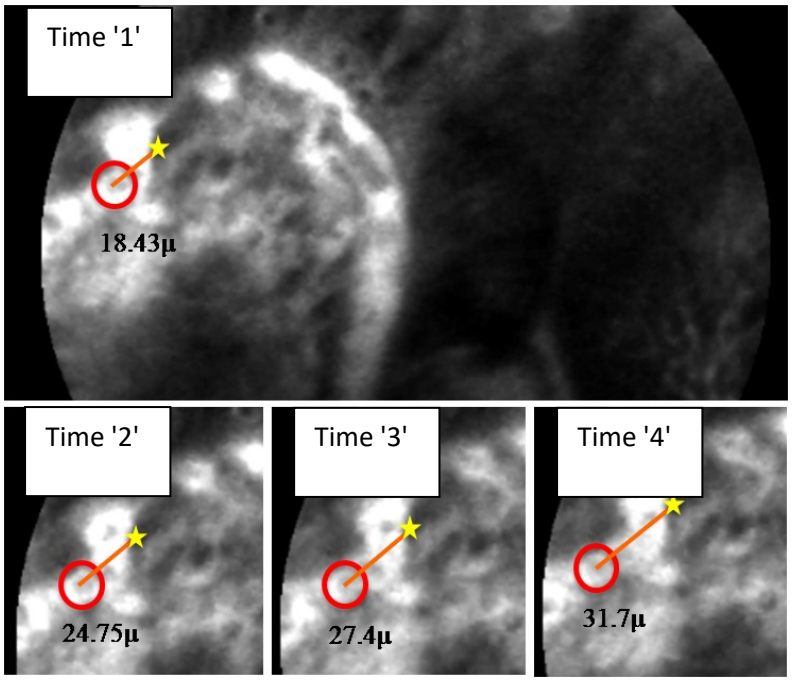


Figure 5.1:

Capillary flow rates in the duodenum of IBD and non-IBD patients were analyzed using probe-based confocal laser endomicroscopy (pCLE). **A.** Image showing a pCLE probe obtaining confocal images of the duodenum during endoscopy. **B.** Representative images of the duodenum obtained using pCLE, showing tracking of a red blood cell and capillary flow rate analysis. The distance between the anatomical reference point (epithelial cell) and the blood cell in the 'time 1' image is 18.43 µ. Capillary flow rate was calculated by measuring the distance traveled by the blood cell between 'time 4' and 'time 1', in relation to the reference point (31.7-18.43=13.37 µm), divided by the time passed (26 ms), as follows:

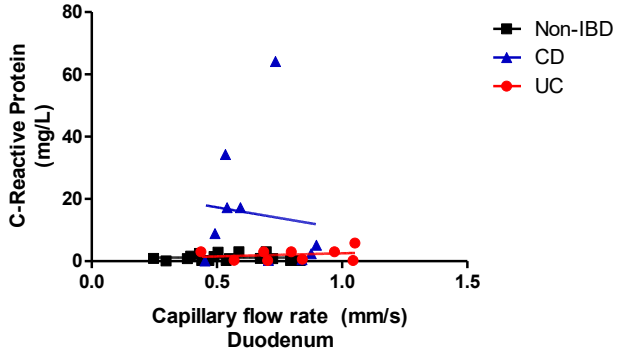
$$\text{Capillary flow rate} = \frac{\text{Distance travelled by blood cell}}{\text{Duration of video sequence}} = 13.37\mu m/26ms = 0.51\mu m/ms \text{ (or mm/s)}$$

C. Capillary flow rates were significantly higher in the duodenum of UC patients [UC: 0.75±0.07; non-IBD controls: 0.57±0.03; CD: 0.65±0.04 (mean±SEM capillary flow rate, mm/s)]; ANOVA, $P< 0.05$, N=22 Non-IBD, 14 CD, and 9 UC patients.

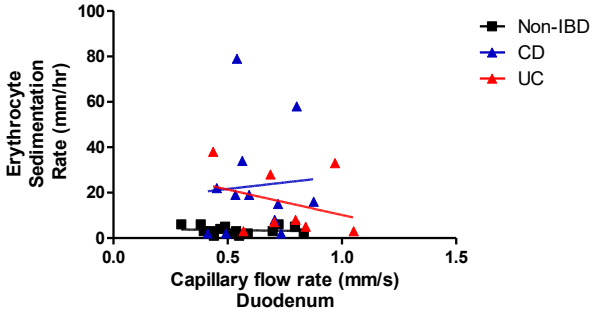
Correlation of capillary flow rates with inflammation: There was no correlation between ESR, CRP, PUCAI, and capillary flow rates in UC patients. Rather, ESR and PUCAI showed a non-significant trend for negative correlation with flow, as did PCDAI. Although our study may not have been large enough to link changes in vascular flow with inflammation, there was no correlation between capillary flow rates and inflammatory markers in non-IBD and UC patients ($P>0.05$, **Fig. 5.2A-C**). This further supports our claim that increased blood flow in UC is present even in the absence of inflammation. While duodenal IL-2 and IL-8 were high in UC patients, there was no correlation between duodenal IL-2 and IL-8 levels and capillary flow rates. None of the patients had active inflammation in the duodenum; however, 10 of 14 CD and 6 of 9 UC patients were found to have active tissue inflammation on mucosal biopsies taken during ileocolonoscopy. In the CD group, 4 patients had inflammation in the TI, 2 in the colon, and 4 in both TI and colon. Three UC and 4 CD patients of the entire cohort were in remission at the time of endoscopy. In a subgroup analysis, we found no difference in the vascular flow rates of patients with active or inactive disease.

Figure 5.2: No correlation between inflammatory markers or disease activity and capillary flow rates

A.



B.



C.

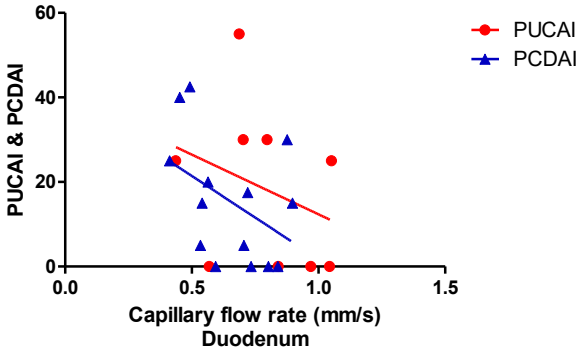


Figure 5.2:

Capillary flow rates were not related to inflammatory markers or disease activity; CRP [**(A),** Non-IBD; Pearson r coefficient = 0.006, P = 0.9. UC; Pearson r coefficient = 0.22, P = 0.56. CD; Pearson r coefficient = -0.11, P = 0.7], Erythrocyte sedimentation rate ESR [**(B),** Non-IBD; Pearson r coefficient = - 0.11, P = 0.6. UC; Pearson r coefficient = -0.3, P = 0.45. CD; Pearson r coefficient = 0.07, P = 0.82], and disease activity indices [PCDAI and PUCAI **(C)**, PCDAI; Pearson r coefficient = -0.43, P = 0.12. PUCAI; Pearson r coefficient = -0.30, P = 0.42.]

Serum hemoglobin and albumin levels:

Increased capillary flow rates could be secondary to anemia and increased cardiac output. Thus, we compared serum hemoglobin levels between groups. There was a trend for lower hemoglobin in IBD patients but this was not statistically significant ($P>0.05$). Hypoprotenemia also affects cardiac output, but there was no difference in albumin levels between groups **(Table 5.1).** None of the patients had hemoglobinopathies, Heinz bodies, red cell defects, or other vascular disorders.

The increased capillary flow was not explained by changes in hemoglobin or albumin as there was no correlation between serum hemoglobin and albumin of UC patients and capillary flow rates (**Fig. 5.3**; Hemoglobin: Pearson r coefficient = 0.32, $P>0.05$; Albumin: Pearson r coefficient = 0.04, $P>0.05$).

Figure 5.3: No correlation between serum hemoglobin and albumin levels of UC patients and capillary flow rates

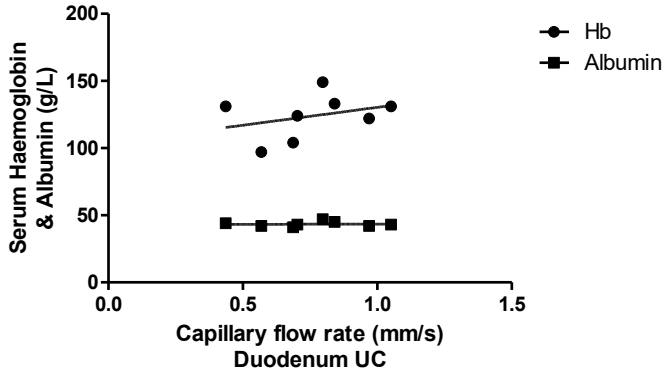


Figure 5.3:

There was no correlation between serum hemoglobin and albumin levels and capillary flow rates in UC patients. Hemoglobin: Pearson r coefficient = 0.32, $P>0.05$. Albumin: Pearson r coefficient = 0.04, $P>0.05$.

5.5 Discussion:

Vascular changes are a hallmark of IBD and have been associated with inflammation (Cromer et al. 2011); the question of whether these changes contribute to, or occur as a consequence of, inflammation remains.

The goal of our study was to identify alterations in microvascular circulation in unaffected areas in IBD patients by quantifying capillary flow rates using pCLE. Our study is the first to measure capillary blood flow rates in IBD patients overall, and specifically in the duodenum, and to report an increase in UC patients, compared to non-IBD. This novel finding in our pilot study is demonstrated in the absence of local inflammation, suggesting that it is not secondary to inflammation. Increased vascular flow was unrelated to inflammatory markers or disease activity (although this was a secondary outcome that our study was not sufficiently powered to test conclusively). The data suggest that vascular changes can occur independent of inflammatory parameters and may represent a fundamental intestinal change in UC patients outside the colon. Capillary flow rates were unchanged in CD patients, which indicate that different pathogenic pathways could be present in UC and CD. Hyperemia in the gut occurs as a result of many stimuli, such as increased tissue oxygen consumption and serotonin, possibly mediated by nitric oxide (Kolios et al. 2004).

Endothelial cells prevent platelet aggregation and excessive leukocyte infiltration in the healthy state, and also maintain low vascular permeability. Changes in blood flow can occur due to sheer stress that can cause vasodilation of arterioles. Vascular flow is

affected by factors secreted by the endothelium, such as prostacyclin, nitric oxide, and endothelium-derived hyperpolarizing factor, which act as vasodilators, or by vasoconstrictors, such as endothelin-1 and thromboxane (Sandoo et al. 2010). Vascular endothelial growth factor (VEGF)-A and B induce angiogenesis in IBD (Scaldaferri et al. 2009). As well, changes in endothelial cells occur in response to inflammation as various cytokines, such as TNF-α, induce VCAM-1 and MAdCAM-1 and promote platelets adherence in IBD. Cytokines involved in IBD also affect gut vasculature, as TNF-α and IL-8 both possess pro-angiogenic properties (Cromer et al. 2011). We did find IL-8 to be elevated in the duodenal biopsies of our UC patients in a previous study (Zaidi et al. 2016), but what triggered this increase is yet unknown. Increased epithelial gaps were found in the same patients in the afore-mentioned study (Zaidi at al. 2016), however, there was no correlation between increased blood flow and the higher density of gaps.

It is tempting to speculate that changes in these (and other, yet unidentified) parameters could potentially be a reason for increased blood flow in the absence of inflammation and could ultimately trigger inflammation. However, this was not directly addressed in this study and such conclusions cannot be made without additional studies with larger cohorts and molecular analysis.

Anemia occurs commonly in IBD due to blood or iron loss, and/or malabsorption (Weiss & Gasche 2010), and can affect cardiac output and blood flow. Tissue hypoxia can result in increased cardiac output, decreased blood viscosity, and peripheral vascular resistance (Biro 1997). To determine whether increased blood flow in the UC patients could be explained by anemia, we compared hemoglobin levels of all groups and did not

find any difference between groups. Flow rates were correlated with hemoglobin levels and we found that variation in flow could not be explained by hemoglobin; in fact, hemoglobin showed a trend for a positive correlation with flow. Having a direct measure of cardiac output would have been a more reliable control, but this was not clinically indicated during the procedures and, therefore, not available. As protein-losing enteropathy is a rare complication of UC (Ungaro et al. 2012), and hypoalbuminemia affects cardiac output, we compared the pre-procedure albumin levels but found no differences between groups.

Our study has several limitations. While patients in the CD group included both newly diagnosed and chronic patients, the UC cohort only had follow-up cases; follow-up cases were on therapy, which could affect our results. Recruitment of treatment-naïve patients, especially with UC, in future studies will be important to address the effect of treatment. Prolongation of the endoscopy procedure with confocal imaging and the requirement of IV injection of fluorescein did, unfortunately, negatively impact recruitment, thus our sample size is small. We would have liked to measure flow rates in inflamed areas as well but had to limit the study to one site to reduce endoscopy time and the need for a second injection of fluorescein. In addition, given that this method has not been described to date in humans, we did not have a reference to compare to. Remarkably, a mouse study has shown a mean intestinal capillary flow rate of 2.6 nl/min, which is almost completely identical to the flow rate we found (after unit conversion and assuming a capillary diameter of 10 μ 0.57 mm/s = 2.686 nl/min) (Foitzik et al. 1999). While capillary flow rates can be successfully quantified using pCLE, we were not

successful in measuring capillary density to assess angiogenesis due to technical challenges. It is possible that the flow changes observed are secondary to changes in the vascular bed, but one would expect increased capillary branching to slow flow, not enhance it.

In conclusion, our study is the first to show quantification of microvascular blood flow in the duodenum of IBD patients, and showed increased capillary flow rates in the unaffected duodenum in UC patients using pCLE. Assessing duodenal blood flow in IBD patients over time in subsequent studies may help determine if disease course or responses to therapy are predicted by blood flow rates. Using this novel application of pCLE technology, future studies need to focus on associating changes in capillary flow rates with underlying vascular pathology in UC to better define disease pathogenesis and possibly identify new treatment targets.

Chapter 6

Dysregulation of TNFAIP3 (A20) is associated with inflammation in pediatric Crohn disease.

6. DYSREGULATION OF TNFAIP3 (A20) IS ASSOCIATED WITH INFLAMMATION IN PEDIATRIC CROHN DISEASE.

6.1 . ABSTRACT:

INTRODUCTION: Inflammatory bowel diseases (IBD), including Crohn disease (CD) and ulcerative colitis (UC), are debilitating intestinal disorders that frequently affect children and young adults. Failure in suppressing inflammation contributes to IBD pathogenesis; identification of factors that regulate inflammation can improve IBD management. A20, also known as tumor necrosis factor α-induced protein 3 (*TNFAIP3*), is a cytoplasmic protein that inhibits NF-κβ-induced inflammation. A20 interacts with A20-binding inhibitor of NF-κβ activation 1 (ABIN-1) and Tax1 binding protein 1 (TAX1BP-1) to attenuate inflammation. IKKβ (inhibitor of nuclear factor kappa-B kinase subunit beta) phosphorylates A20 and stabilizes it. A20 expression is reduced in adult CD patients. We hypothesized that dysregulation of A20 contributes to uncontrolled inflammation in pediatric IBD.

METHODS: A total of 39 patients were included in the study (14 non-IBD controls, 15 CD, and 10 UC patients). Gene expression of *A20, IKKβ, ABIN-1,* and *TAX1BP1*, as well as A20 protein and cytokine levels in the TI of IBD and non-IBD patients were analyzed and compared to disease markers/activity. *A20* gene expression and protein levels in T-84

cells and *ex-vivo* biopsies of patients after treating with TNF-α or *Escherichia coli* strains LF-82 and HB101 were analyzed.

RESULTS: TNF-α level and *A20* gene expression were significantly elevated in the TI of pediatric CD patients compared to non-IBD and UC. In contrast, A20 protein levels and *ABIN-1* expression were significantly low, *IKKβ* was unchanged, and *TAX1BP1* expression was high. *A20* gene expression positively correlated with biopsy TNF-α levels, serum C-reactive protein, and erythrocyte sedimentation rates in CD patients. Polar findings regarding *A20* gene expression and protein levels in CD biopsies led us to assess the possibility of bacterial effects on A20 stability. Infection with *E. coli,* strain LF82 triggered A20 expression in TI biopsies from CD patients and T84 cells, but did not cause an increase in A20 protein levels.

CONCLUSIONS: Our study reports a possible mechanism for failure of A20 to down-regulate inflammation in pediatric CD. We have shown, for the first time, a unique signature profile in pediatric CD patients in the form of high *A20* and *TAX1BP1*, low *ABIN-1*, unchanged *IKKβ* expression levels, and low A20 protein levels. The discrepancy between *A20* expression and protein levels is possibly due to the concomitant lower expression of *ABIN-1*, and instability of A20 protein due to lack of post-translational phosphorylation related to lower expression of *IKKβ*. *E. coli* strain LF82 augments *A20* expression, but not A20 protein, suggesting that microbes could hinder the capacity of A20 to limit inflammation. Thus, factors affecting A20, including microbes, negatively

impact the A20 protein's anti-inflammatory action in pediatric CD, and contribute towards unremitting inflammation.

6.2 Introduction:

Inflammatory bowel diseases (IBD), including both Crohn disease (CD) and ulcerative colitis (UC) are chronic diseases of the gastrointestinal tract that severely affect children and young adults (Eszter et al. 2014; Benchimol et al. 2014). The etiology of IBD is not clearly understood, but likely involves an uncontrolled immune response to environmental/microbial stimuli in genetically susceptible hosts.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ), a pro-inflammatory nuclear transcription factor, plays a central role in innate immunity and imbalances in NF-κβ-associated factors can lead to chronic inflammation and altered immunity (Oeckinghaus et al. 2011; Hayden & Ghosh 2008), including in IBD (Atreya et al. 2008). A20, also known as, tumour necrosis factor (TNF) α-induced protein 3 (TNFAIP3), is a cytoplasmic inhibitor of NF-κβ-induced inflammation and is triggered by NF-κβ activation.

A20 plays a critical regulatory role in gut barrier protection during inflammation, as demonstrated by increased apoptosis and damaged intestinal epithelium after TNF-α treatment of intestinal epithelial cells-specific A20 knock-out mice (Vereecke et al. 2010). Genome-wide association studies (GWAS) have linked A20 with IBD (Ma & Malynn 2012). In adult IBD patients, A20 profiling has shown varying correlations with disease phenotype and severity. A20 expression is low in the colonic and terminal ileum (TI) mucosa (Arsenescu et al. 2008) and increased in colonic biopsies of adult UC, but not in CD patients (Fernandes et al. 2016b).

A20's anti-inflammatory effects are facilitated through interactions with other proteins, such as A20-binding inhibitor of NF-κβ activation 1 (ABIN-1) (Mauro et al. 2006) and Tax1 binding protein 1 (TAX1BP-1) (Shembade et al. 2011; Verstrepen et al. 2011). ABIN-1 attaches A20 to the IKK/NEMO complex and assists it exerting its deubiquitinating activity (Mauro et al. 2006). *ABIN-1* expression depends on NF-κβ, and it also inhibits TNF-α-mediated apoptosis (Verstrepen et al. 2009). In mice, ABIN-1 was found to attach A20 to NEMO/IKKγ and facilitate deubiquitination (Mauro et al. 2006). TAX1BP1 inhibits NF-κβ inflammation (Shembade et al. 2011) and helps A20 regulate its deubiquitinating activities by recruiting A20 to the polyubiquitin chains to break and interrupt the IKK complex assembly (Verstrepen et al. 2011). IKKβ phosphorylates A20 at the Serine 381 (S381) site, which stabilizes A20 and attenuates NF-κβ signalling (Hutti et al. 2007). Studies in mice have shown that A20 in the phosphorylated state cleaves Lys-63-linked polyubiquitin chains, and inhibits NF-κβ signalling (Wertz et al. 2015).

Regulating or minimizing chronic inflammation is a major goal of the current IBD treatment regimen, as most current treatments are immunosuppressant. Sustained inflammatory regulation is critical for optimizing treatment and hence, identification of factors that regulate inflammation is critically needed to improve IBD management and may reduce the need for non-selective immunosuppressant medications. Given the fact that A20 can inhibit inflammation, and since TNF-α-associated intestinal inflammation is a critical factor in IBD pathogenesis, we explored the correlation between TNF-α production and A20 expression.

In children, IBD severely impacts development and has a more severe phenotype. Long-term therapies and co-morbidities robustly impact immunoregulatory pathways. Thus, it is essential to investigate the inflammatory paradigms in children in order to gain early insight into the gut inflammatory network, which can help towards an "immune-tailored" therapeutic approach.

We hypothesized that dysregulation of A20 contributes to uncontrolled inflammation in IBD and is mediated by factors anchoring and stabilizing A20. In this study, we looked at the gene expression of *A20, IKKβ, ABIN-1, and TAX1BP1*, and A20 protein levels in TI biopsies of IBD and non-IBD patients. We also assessed the effects of *Escherichia coli* strains on A20 regulation in T-84 cells and *ex-vivo* TI biopsies of CD patients.

We have shown, for the first time, a unique signature profile in pediatric CD patients in the form of high *A20* expression levels and low A20 protein levels. The discrepancy in A20 gene and protein levels is possibly due to the concomitant lower expression of ABIN-1, offering a potential explanation for the failure of A20 to down-regulate inflammation. *E. coli* strain LF82 augments A20 expression, but not A20 protein, suggesting that microbes could hinder the capacity of A20 to limit inflammation.

6.3 Methods:

Patients: The study included samples from a prospectively-collected cohort of pediatric IBD and non-IBD patients undergoing gastroscopy and colonoscopy through the Edmonton Pediatric IBD Clinic (EPIC), Stollery Children's Hospital, University of Alberta. Prior to the study, ethics approval from the University of Alberta Research Ethics Board (Study ID Pro00023820) was obtained. The control group included non-IBD patients undergoing endoscopy for intestinal symptoms, such as, diarrhea, abdominal pain, etc., but they were excluded upon any abnormal endoscopic or histological mucosal finding. The IBD group included new and previously diagnosed CD and UC patients, based on the Porto criteria (Levine et al. 2014). Detailed clinical, laboratory, endoscopic, and histologic findings were prospectively recorded form the patient charts. All patients were given Picosalax® (sodium picosulphate with magnesium citrate) prior to the procedure for bowel preparation. After being administered general anesthesia with propofol by a pediatric anesthesiologist, endoscopies were performed by pediatric gastroenterologists. Biopsies were obtained from the TI and snap-frozen at -80ºC within 30 min for RNA and protein analysis, or placed in cold Hank's Balanced Salt Solution (HBSS) for *ex-vivo* experiments.

Total RNA extraction and reverse transcription PCR: TI biopsies were homogenized in Trizol (Life Technologies, Burlington, ON, Canada) and RNA was extracted using a Qiagen RNeasy Kit (Qiagen, Mississauga, ON, Canada). After quantification with Nano-

Drop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), 500 ng of the RNA were reverse transcribed using the Quantitect Reverse Transcription kit (Qiagen, Mississauga, ON, Canada) following the manufacture's protocol. The cDNA was used to conduct qRT-PCR on 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR cycle settings were as follows: polymerase activation at 50ºC for 2 minutes, initial denaturing of template DNA for 10 minutes at 94ºC, 35 denaturing cycles for 15 seconds at 95ºC, annealing for 15 seconds at 58ºC, and elongation for 30 seconds at 72ºC. Relative quantification was done to compare changes in the gene expression amongst non-IBD and IBD patients. *Glyceraldehyde phosphate dehydrogenase* (*GAPDH*) was used as a housekeeping gene for relative quantification of the transcript amount (Bertiaux-Vandaële et al. 2011). Gene expressions of *IKKβ* (Roche Diagnostics, Canada); *TNF-α-Induced Factor 3* (*TNFAIP3*; also known as *A20*) (J. Liu et al. 2013b); *ABIN-1* (Adrianto et al. 2012); and *TAX1BP1* (Gröschl et al. 2013) were calculated using the $2^{-\Delta\Delta CT}$ method.

A20 Protein Quantification: Biopsies were processed for protein and cytokine analysis as described in section **Methods 3.3**. A20 protein levels in the biopsies and T84 cells were quantified using the A20 ELISA kit (Biomatik, Cambridge, Ontario, Canada), following the manufacturer's instructions.

Cytokine Analysis: Cytokine analysis of the TI biopsies of the patients was done using the V-Plex pro-inflammatory panel 1 kit (Mesoscale Diagnostics, Rockville, MD, USA).

Cytokine profiles of interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α were analyzed with this kit and reported relative to standard curves, according to the manufacturer's instructions.

Immunofluorescence Microscopy: Biopsy samples obtained from the TI were mounted in OCT tissue embedding media (Leica Biosystems, Concord, Ontario, Canada). Tissue sections were cut 5 µm thick by cryotome, embedded on slides, and slides were frozen at -80ºC. Slides were then fixed for 10 minutes in ice-cold acetone, washed 3x in 1x PBS, and incubated in blocking buffer (10% NGS) for 1 hour at room temperature. After washing again in PBS, slides were incubated overnight at 4 ºC with anti-TNFAIP3 (A20) antibody (Abcam, Toronto, Ontario, Canada) at a dilution of 1:200. Slides were then rinsed again in PBS and stained with Alexa 546 goat anti-mouse secondary antibody (Thermo Fisher Scientific, Mississauga, Ontario, Canada) for 1 hour after being diluted at 1:300. Slides were incubated with 4',6-diamidino-2-phenylindole (DAPI; Thermofisher Scientific, Mississauga, Ontario, Canada) for 1 minute after rinsing in PBS, and mounted with Fluorsave (Millipore, Etobicoke, Ontario, Canada). Slides were viewed with the Zeiss Axio Observer.Z1 microscope with ZEN Imaging software (Carl Zeiss Canada Ltd., North York, Ontario, Canada). Immunofluorescence staining of the tissues was observed using the 561 nm laser filter for A20 staining (red dye) and the 405 nm laser for DAPI (blue dye). All images were captured at 60x.

Cell culture:

Colorectal cell line, T-84, obtained from ATCC, was chosen for *in vitro* experiments as it expresses A20 (Li et al. 2013; El-Etr et al. 2004). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and 1% penicillin/streptomycin at 37ºC in 5% CO2. The medium was changed every alternate day and cells were passaged every week when confluent. Cells were seeded in 24-well plates at a density of 1 x 10^6cells per cm^2 and grown to confluence for 5 days for infection experiments.

Bacterial strains and reagents: Adherent invasive *E. coli*, strain LF82 (Darfeuille-Michaud et al. 2004) and *E. coli*, lab strain HB101 were used for the *ex vivo* biopsy and T-84 cells assays. Both strains were cultured in Luria-Bertani (LB) broth overnight at 37º C prior to the inoculation with the biopsies and cell line. TNF-α, 1 ng/ml (Fish et al. 1999) and LPS, 10 µg/ml (Cario et al. 2000) were used as positive controls to stimulate A20.

Infection of T-84 cells: T-84 cells were inoculated with either the *E. coli* strains, LF82 and HB101 (suspensions containing $1x10^8$c.f.u), TNF-α (1 ng/ml), or LPS (100 ng/ml) in duplicates. Infected cells were incubated for 4 hours at 37º C, 5 % CO_2, after which epithelial cells were collected and stored at -80º C for RNA and protein analyses. RNA was extracted using Qiagen RNeasy Kit (Qiagen, Mississauga, ON, Canada), and samples were processed for protein extraction as described in section **'Methods 3.3'**.

Ex vivo infection of human TI biopsies: *Ex vivo* infection was performed using methods described previously, with minor modifications (MacCallum et al. 2006). TI biopsies obtained from non-IBD and CD patients during endoscopy were immediately placed in HBSS on ice. Biopsies were then incubated with DMEM containing 10% FBS and penicillin/streptomycin (1mg/ml) for one hour to kill resident bacteria. Biopsies were then washed, medium was changed to antibiotic-free, and samples inoculated with either the *E. coli* strains, LF82 or HB101, or TNF-α at the same concentrations as for the T-84 cells for 10 hours. mRNA [using the Qiagen RNeasy Kit (Qiagen, Mississauga, ON, Canada)], and protein were extracted from the samples (as described in section **'Methods 3.3')** at the end of the incubation period and stored at -80º C for future analysis.

NF-κβ ELISA: Cellular extraction prior to conducting the assay was done using the Cell Extraction Buffer (Invitrogen, Canada). To quantify NF-κβ stimulation with treatment of the *ex vivo* biopsies and T-84 cells, an ELISA of the NF-κβ-65 transcription activation domain was conducted using an NF-κβ-65 kit (Invitrogen, Burlington, Ontario, Canada), following the manufacturer's instructions.

Statistical analysis: Data were analyzed using Graph Pad Prism (San Diego, CA, USA). Student's *t*-test was used to compare the cohort and treatment groups. The RQ values were analyzed using the 2ΔCT method. Correlation analysis was done using Pearson's correlation coefficient. $P<0.05$ was considered statistically significant.

6.4 Results:

Patients: A total of 45 patients were screened and consented for this study. Complete data are available for 39 patients, including 14 non-IBD controls, 15 CD, and 10 UC patients (4 controls and 2 UC patients were excluded as A20 expression could not be detected). Of the CD cohort, 7 patients were newly diagnosed and 8 were previously diagnosed. Of the UC patients, 9 were follow-up cases and one was a new case. Patient characteristics are described in **Table 6.1**.

Table 6.1: Patient Characteristics

Demographic	***Non-IBD (n=14)***	***CD (n= 15)***	***UC (n= 10)***	***'P' value***
Sex (m/f)	8/6	7/8	4/6	
New onset	N/A	7	1	
Follow-up	N/A	8	9	
Age (mean ± SEM)	12 ± 1.8	12.85 ± 1.1	13.44 ± 1.1	0.25
CRP* (<8 mg/L)		20.56 ± 6.7	3.1 ± 0.9	0.8
ESR* (0-15 mm/hr)		26 ± 7.0	9.5 ± 6.3	0.74
PCDAI /PUCAI* (<5)		21.36 ± 3.2	19.9 ± 4.6	
Treatments at time of endoscopy				
5-aminosalicylic acid – N (%)	-	1 (6%)	1 (10%)	
Sulfasalazine	-	1 (6%)	1 (10%)	
Prednisone	-	1 (6%)	1 (10%)	
Azathioprine	-	2 (13%)	2 (20%)	
Methotrexate	-	1 (6%)	1 (10%)	
Infliximab	-	4 (26%)	3 (30%)	

IBD, inflammatory bowel diseases; CD, Crohn disease; UC, ulcerative colitis; f, female; m, male. * All data reflect status around time of endoscopy.

A20 expression is increased in the TI of CD patients and correlates with TNF-α and inflammatory markers.

Both A20 gene expression and TNF-α levels were significantly higher in the TI tissue of CD patients, compared to non-IBD and UC patients **(Fig. 6.1A and 1B, respectively; P<0.05)** and were positively correlated **(Fig. 6.1C).** All other measured cytokines showed no significant difference, suggesting that A20 expression is selectively increased in the presence of high TNF-α, as expected. A20 gene expression in TI biopsies also correlated with the objective serum inflammatory markers CRP and ESR, but not with the mostly symptom-based PCDAI **(Fig. 6.1D).**

Figure 6.1: A20 expression and TNF-α levels are high in the TI of CD patients, and A20 expression correlates with TNF-α levels and inflammatory markers.

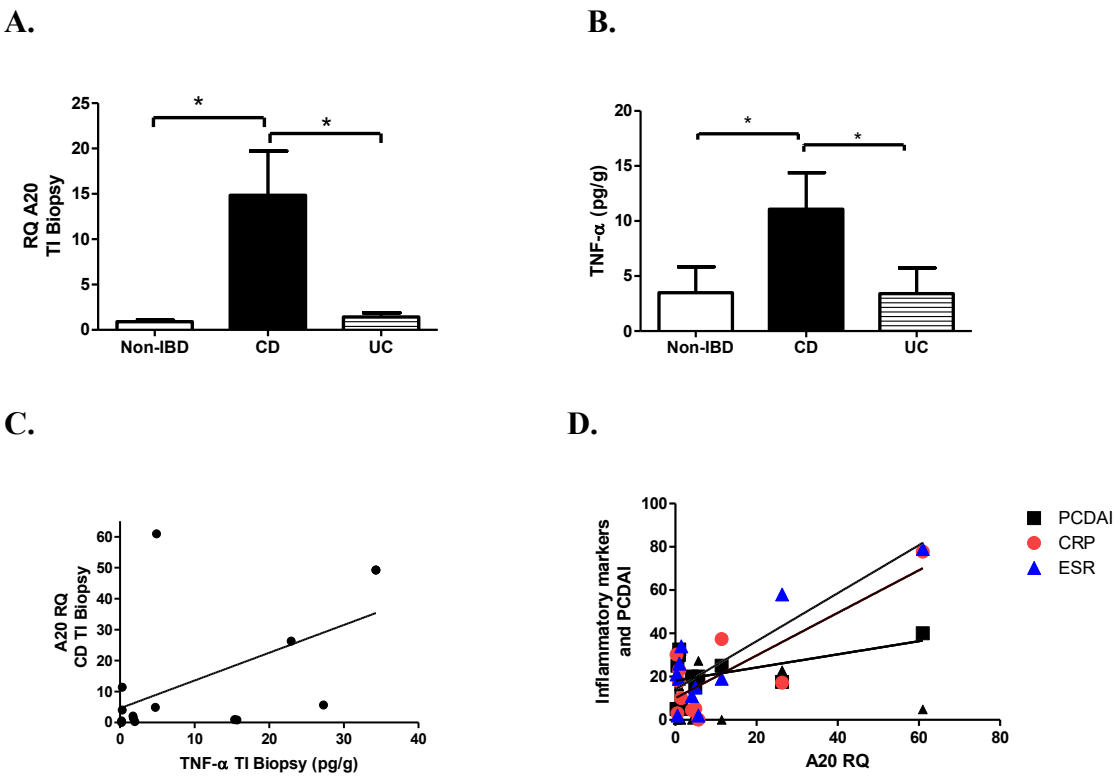


Figure 6.1:

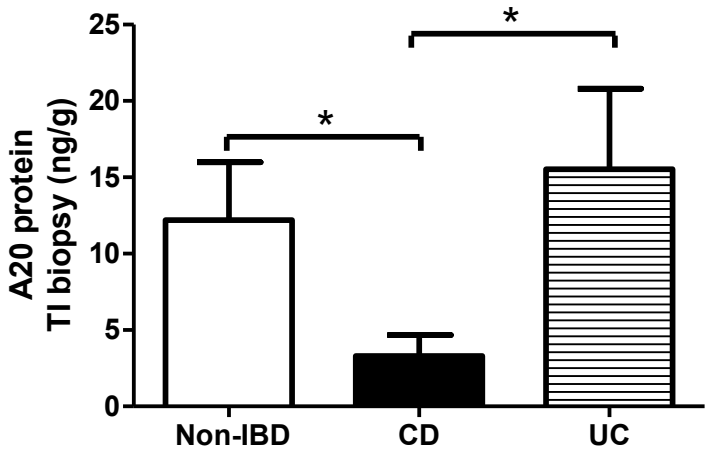
Quantification of *A20* expression and TNF-α levels was conducted on TI biopsies of patients. **A.** *A20* expression was significantly higher in CD patients as compared to non-IBD and UC patients (non-IBD: 0.96 ± 0.15; CD: 14.85 ± 4.7; UC: 1.1 ± 0.46, ANOVA, $P < 0.05$). **B.** Cytokine analysis was performed on TI biopsies of patients**.** TNF-α levels were significantly higher in the TI of CD patients as compared to non-IBD and UC patients (non-IBD: 3.5 ± 2.4; CD: 11.1 ± 3.3, UC: 3.4 ± 2.3 pg/g. Student's *t*-test: $P < 0.05$). **C.** *A20* expression in TI biopsies of CD patients correlated positively with biopsy TNF-α levels (Pearson 'r': 0.5, $P < 0.05$). **D.** *A20* gene expression in TI biopsies is positively correlated with serum CRP and ESR (CRP; Pearson r coefficient = 0.80, $P < 0.05$. ESR; Pearson r coefficient = 0.87, < 0.05). *A20* expression was not related with PCDAI (Pearson r coefficient = 0.5, $P = 0.10$).

A20 protein levels are reduced and A20-regulating factors are altered in TI biopsies from CD patients.

Surprisingly, tissue levels of A20 protein, as quantified by ELISA, were significantly lower in CD patients compared to non-IBD and UC patients, despite the higher gene expression **(Fig. 6.2A).** To assess potential explanations for the discrepancy between A20 gene expression and protein levels, we next analyzed expression of A20-regulating factors. *IKKβ* expression was unchanged amongst non-IBD and CD patients **(Fig. 6.2C)**. *TAX1BP1* expression was significantly higher in CD cases **(Fig. 6.2D; P<0.05)**, whereas *ABIN-1* expression was significantly lower in CD patients relative to non-IBD cases **(Fig. 6.2E; P<0.05)**. These findings suggest that alterations in factors regulating A20 could explain the discrepancy between *A20* gene expression and protein levels.

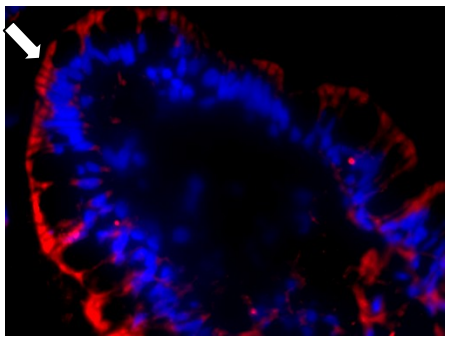
Figure 6.2: A20 protein levels are reduced and A20-regulating factors are altered in TI biopsies from CD patients.

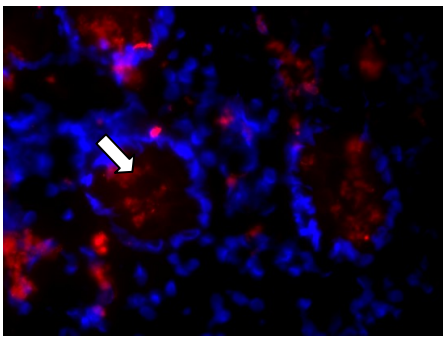
A.



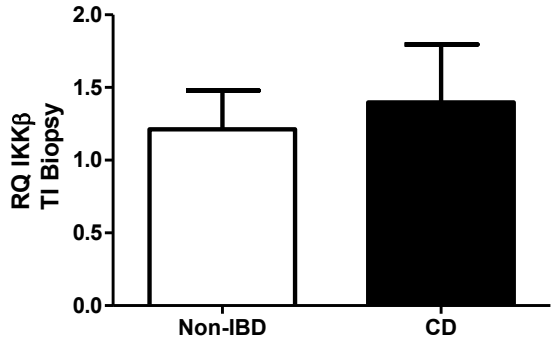
B.

Non-IBD **CD**

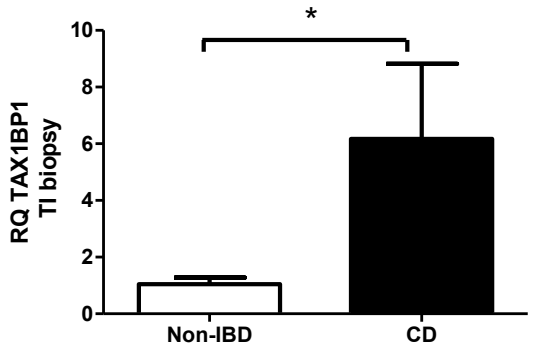




C.


RQ IKKβ
TI Biopsy
2.0
1.5
1.0
0.5
0.0
Non-IBD
CD

D.


*
RQ TAX1BP1
TI biopsy
10
8
6
4
2
0
Non-IBD
CD

E.

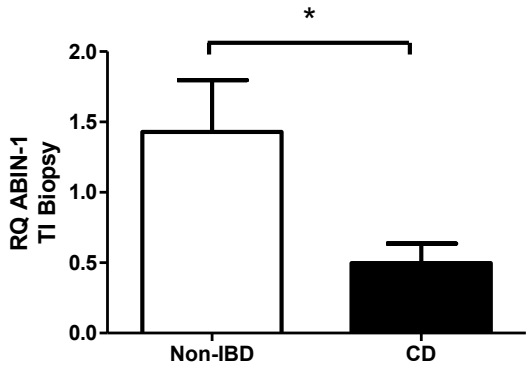

*
RQ ABIN-1
TI Biopsy
2.0
1.5
1.0
0.5
0.0
Non-IBD
CD

Figure 6.2:

A20 protein levels were quantified in the TI biopsies of patients with ELISA. **A.** A20 protein levels in TI biopsies of CD patients were significantly lower than those of non-IBD and UC patients (non-IBD: 12.19 ± 3.8, CD: 3.30 ± 1.3, UC: 15.5 ± 5.2 ng/g. Student's *t*-test: $P < 0.05$). **B.** Representative images of A20 immunofluorescence in TI of non-IBD and CD patients show the presence of A20 in the tissue. Red colour indicates A20 antibody and blue staining is for DAPI. **C.** *IKKβ* expression was unchanged between non-IBD and CD patients (non-IBD: 1.21 ± 0.3; CD: 1.3 ± 0.4). **D.** *TAX1BP1* expression was significantly higher in CD patients as compared to non-IBD cases (non-IBD: 1.04 ± 0.23; CD: 6.17 ± 2.6. Student's *t*-test: $P < 0.05$). **E.** *ABIN-1* expression was significantly lower in CD patients as compared to non-IBD cases (non-IBD: 1.4 ± 0.4; CD: 0.4 ± 0.3).

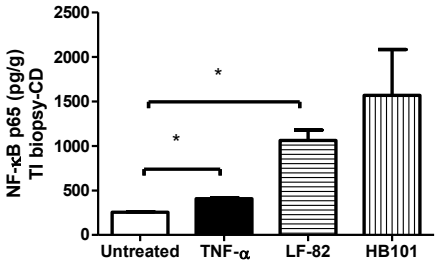
***E. coli* LF82 treatment increased A20 expression but does not affect A20 protein levels in T84 cells.**

As we found a striking discrepancy between *A20* gene expression and protein levels in the TI of CD patients, and since bacteria are altered in IBD and could impact immune responses (Alipour et al. 2015), we hypothesized that bacteria could possibly be a contributing factor that affects A20 stability and action (Zargar et al. 2015) and explored this possibility by conducting infection experiments on a T84 cell culture model and on *ex vivo* biopsies.

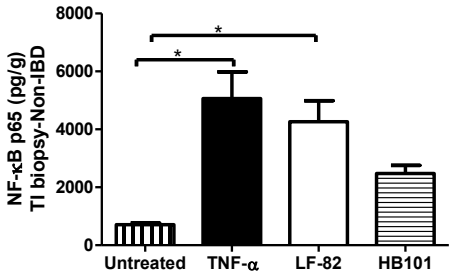
The outcome we chose to confirm activation of the NF-κβ pathway was quantification of translocation of the p65 subunit (we confirmed that bacterial infection and incubation with the positive control, TNF-α, did indeed lead to activation of NF-κβ**; Fig. 6.3, Student's *t*-test; P<0.05).**

Figure 6.3: NF-κβ is activated by bacterial infection in *ex vivo* biopsies and T84 cells

A.



B.



C.

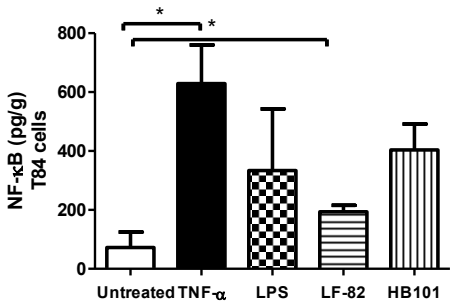
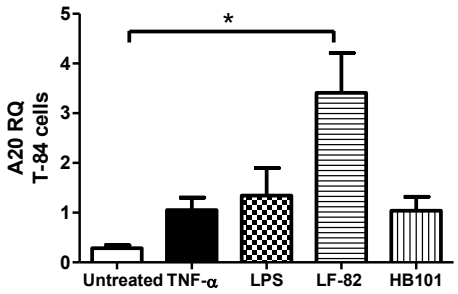


Figure 6.3: NF-κβ p65 was stimulated by TNF-α and bacteria after incubation with patient biopsies and T-84 cells and showed increased nuclear translocation as compared to untreated samples. **A**. NF-κβ p65 levels in treated CD biopsies. **B**. NF-κβ p65 levels in treated non-IBD biopsies. **C**. NF-κβ p65 levels in treated T-84 cells.

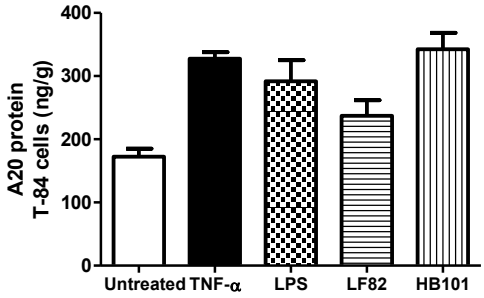
As adherent-invasive *E. coli* (AIEC) are particularly linked to CD patients (Strober 2011), we incubated T-84 cells with the AIEC strain LF82, or, alternatively, the commensal *E. coli* HB101, as well as TNF-α, and LPS as positive controls. *A20* gene expression was significantly higher in LF82-treated vs. untreated cells; there was a modest and non-significant increase in *A20* expression with treatment with HB101, TNF-α, and LPS **(Fig. 6.4A),** [LF82: 3.4 ± 0.8, HB101: 1 ± 0.27. TNF-α: 1.05 ± 0.24, LPS: 1.34 ± 0.53, untreated: 0.28 ± 0.05]. Notably, A20 protein levels after inoculation with LF82 appeared to be lower than those of other treatment groups, but this difference did not reach statistical significance **(Fig. 6.4B, P>0.05).** There were trends for increased expression of other A20-regulating genes, but none reached significance **(Fig. 6.4C-E)**.

Figure 6.4: *E. coli* LF82 treatment increases A20 expression but not A20 protein levels in T84 cells

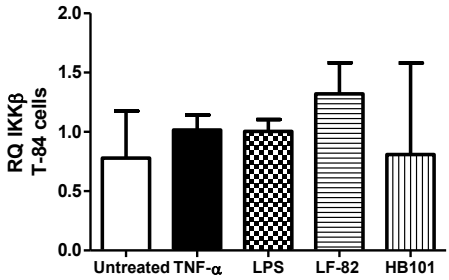
A.



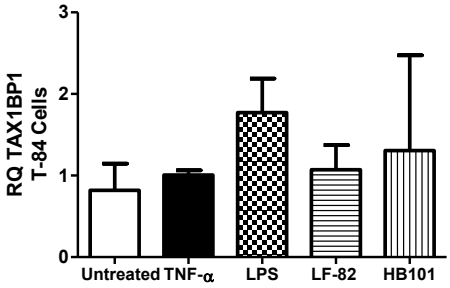
B.



C.



D.



E.

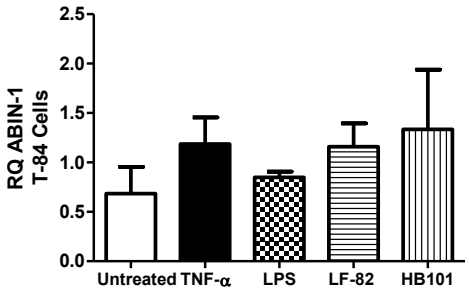


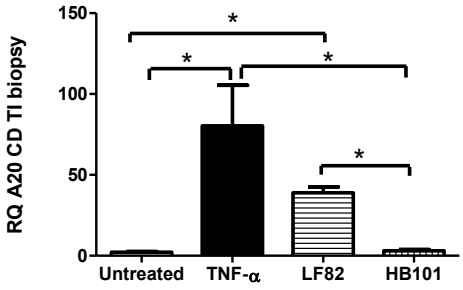
Figure 6.4: T84 cells were subjected to treatment either with the *E. coli* strains, LF82 or HB101 or TNF-α or LPS, as positive controls. **A.** A20 expression after treatment with LF82 was significantly higher than untreated cells (LF82: 3.5 ± 0.8; TNF-α: 1.05 ± 0.2; LPS: 1.3 ± 0.5; HB101: 1.03 ± 0.2; untreated: 0.28 ± 0.05. Student's *t*-test, $P<0.05$ n=7). **B**. A20 protein levels did not differ significantly amongst all groups. **C.** IKKβ expression after the treatments was not significantly affected by the treatments (LF82: 1.4 ± 0.3; TNF-α: 1.0 ± 0.1; LPS: 1.0 ± 0.9; HB101: 0.8 ± 0.7; untreated: 0.7 ± 0.3). **D.** TAX1BP1 expression was not significantly changed after the treatments (LF82: 1.0 ± 0.3; TNF-α: 1.0 ± 0.05; LPS: 1.8 ± 0.4; HB101: 1.3 ± 1.1; untreated: 0.8 ± 0.3). **E.** ABIN-1 expression did not change significantly (LF82: 1.15 ± 0.2; TNF-α: 1.18 ± 0.2; LPS: 0.84 ± 0.05; HB101: 1.3 ± 0.6; untreated: 0.6 ± 0.2).

LF82 infection increased A20 gene expression but did not alter A20 protein levels and ABIN-1 expression in TI biopsies of CD patients:

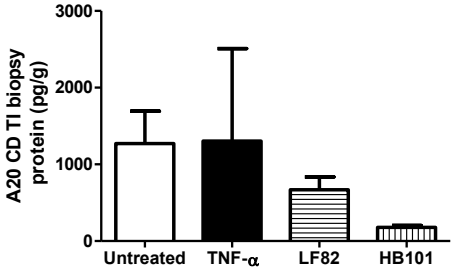
In order to confirm the *in vitro* findings with T84 cells using patient samples, we collected fresh TI biopsies during endoscopy and treated them with *E. coli* strains LF82 or HB101 or TNF-α to define the effects of microbes on A20 regulation. Supporting our findings in biopsies and cell culture, treatment of TI biopsies taken from CD patients with LF82 and TNF-α caused a significant increase in A*20* expression, compared to untreated biopsies and those treated with commensal lab strain HB101 **(Fig. 6.5A, P<0.05)**. A20 protein levels did not increase after treatment with LF82, compared to TNF-α and the untreated biopsies **(Fig. 6.5B)**. This indicates a similar trend as that observed in TI biopsies **(Fig. 6.1A and 6.2A)**, although the difference there reached significance. *IKKβ* expression was also higher after treatment with LF82 compared to the untreated group **(Fig. 6.5C, P<0.05)**. *TAX1BP1* expression increased moderately with treatment by both *E. coli* strains and TNF-α **(Fig. 6.5D, P>0.05)**. While TNF-α treatment did cause a moderate increase in *ABIN1* expression, LF82 did not affect this factor **(Fig. 6.4E, P >0.05)**.

Figure 6.5: LF82 treatment of biopsies from CD patients increased A20 gene expression but not A20 protein levels and ABIN-1 expression.

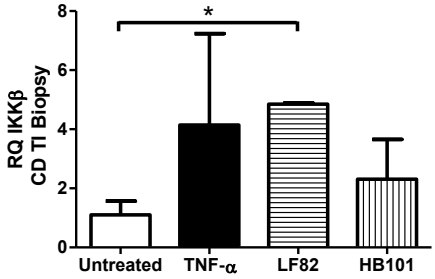
A.



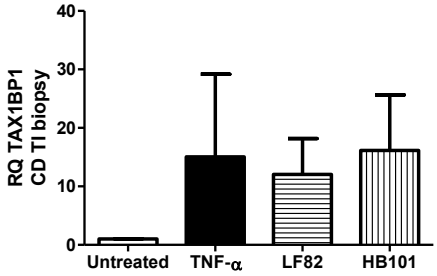
B.



C.



D.



E.

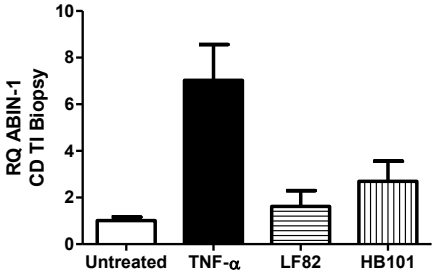


Figure 6.5: TI biopsies from CD patients were incubated with either the *E. coli* strains LF82 or HB101 or with TNF-α. **A.** Treatment with LF82 and TNF-α caused a significant increase in *A20* expression as compared to the untreated biopsies. Although HB101 treatment also increased *A20* expression, this was significantly less than with the other treatments (LF82: 38.9 ± 3.5; TNF-α: 80.3 ± 25.1, HB101: 3.1 ± 0.75; untreated: 2.1 ± 1.1, Student's *t*-test, $P< 0.05$). **B.** A20 protein levels did not differ significantly amongst all groups. **C.** *IKKβ* expression increased after treatment with LF82 compared to the untreated group (LF82: 4.8 ± 0.03; TNF-α: 4.14 ± 3; HB101: 2.3 ± 1.3; untreated: 1.1 ± 0.4, Student's *t*-test, $P<0.05$). **D.** *TAX1BP1* expression increased moderately by treatment by both *E. coli* strains and TNF-α (LF82: 12 ± 6.1; TNF-α: 15 ± 14.1; HB101: 16.1 ± 9.4. N=4, Student's *t*-test : $P< 0.05$). **E.** While TNF-α treatment did cause a moderate increase in *ABIN1* expression, LF82 did not (LF82: 1.5 ± 0.6; TNF-α: 7.3 ± 4.7; HB101: 2.6 ± 0.8; untreated: 1.0 ± 0.15, Student's *t*-test, $P >0.05$).

Supporting the specificity of our findings, infection or TNF-α treatment of TI biopsies of non-IBD patients did not alter gene expression or A20 protein levels **(Fig. 6.6)**. Of note, the basal expression level of all genes was low in biopsies from non-IBD cases, and the treatments caused a very slight, almost negligible increase in the A20 expression level.

Our *in vitro* and *ex vivo* results further validate the findings in TI biopsies of CD patients, suggesting that inflammation increases A20 expression without an increase in A20 protein levels, possibly through LF82-mediated attenuation of ABIN-1. This is important as ABIN-1 acts as a chaperone to A20, helping it to the IKK/NEMO complex, and it's downregulation/absence attenuates A20 activity (Mauro et al. 2006).

Figure 6.6: Gene expression profile and A20 protein levels in e*x vivo* treated TI biopsy of non-IBD patients were not altered by bacterial infection

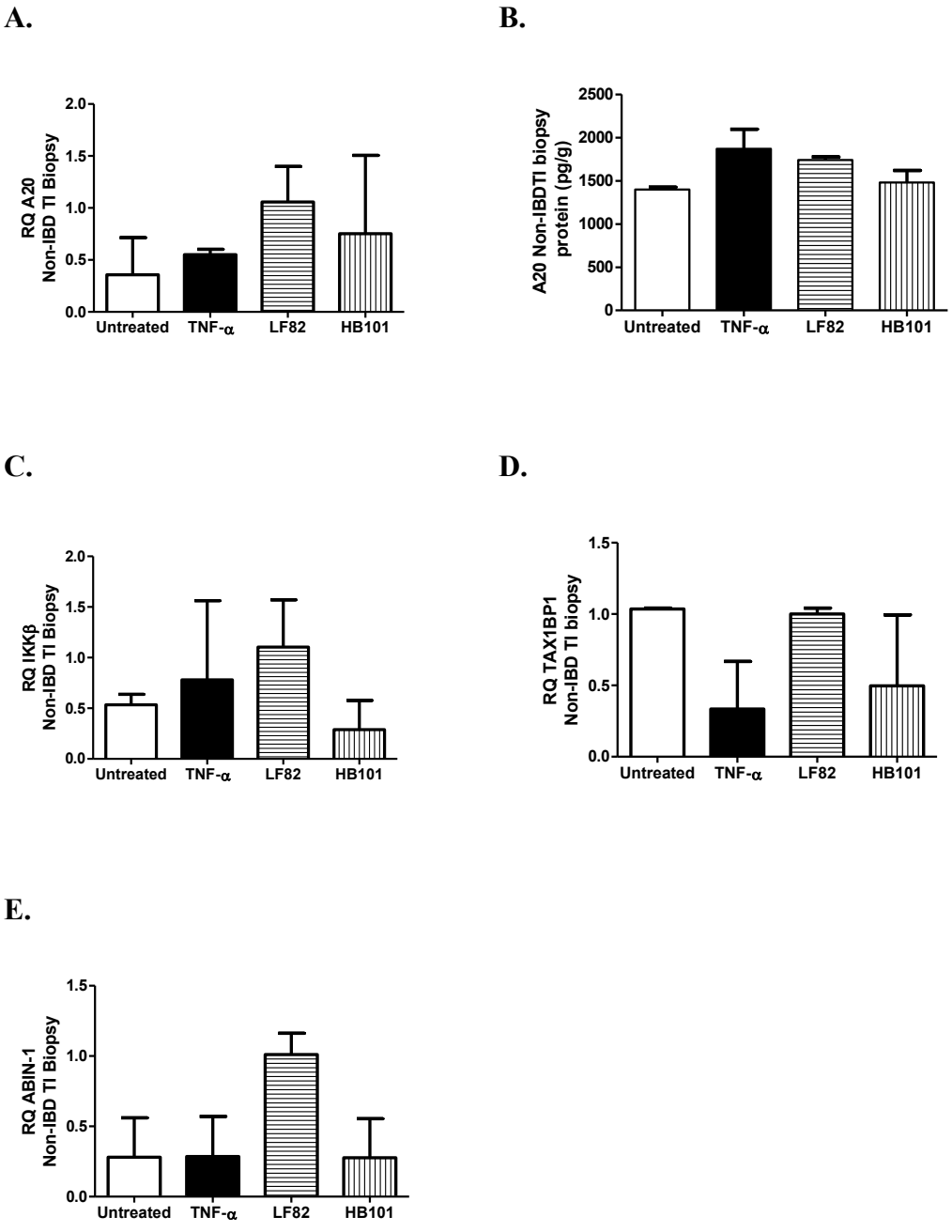


Figure 6.6:

Although slightly increased with treatments, there was not a significant change in the expression profile of all genes. **A.** *A20* expression in TI biopsies after treatment (LF82: 1.05 ± 0.3; TNF-α: 0.55 ± 0.05; HB101: 0.75 ± 0.7; untreated: 0.35 ± 0.3). **B.** A20 protein levels in TI biopsies of non-IBD patients (LF82: 1741 ± 35.7; TNF-α: 1870 ± 226.1; HB101:1480 ± 140.4; untreated: 1399 ± 26.87). **C.** *IKKβ* expression in TI biopsies (LF82: 1.1 ± 0.4; TNF-α: 0.78 ± 0.7; HB101: 0.28 ± 0.2; untreated: 0.53 ± 0.10). **D.** *TAX1BP1* expression in TI samples (LF82: 1.0 ± 0.04; TNF-α: 0.33 ± 0.3; HB101: 0.5 ± 0.4; untreated: 1.0 ± 0.005). **E.** *ABIN-1* expression in TI biopsies (LF82: 1.0 ± 0.15; TNF-α: 0.28 ± 0.2; HB101: 0.27 ± 0.2; untreated: 0.28 ± 0.2).

6.5 Discussion:

The inability to control inflammation is a central feature of IBD, which poses a great challenge towards establishing safe and effective treatment. Modulation of inflammation-associated factors with potential to suppress inflammation in a specific way is a critical step to optimize treatment strategies.

Amongst the perturbed cytokine balances in IBD, the presence of high tissue TNF-α in CD patients has been a consistent finding (Murch et al. 1993; Reimund et al. 1996). Therefore, it is important to identify factors that naturally control TNF-α. A20 is an important and 'natural' inhibitor of TNF-α-induced NF-κβ inflammation (Catrysse et al. 2014). Thus, our objective was to study A20 and its associated factors in pediatric CD with the goal of finding novel approaches to control inflammation.

Gene expression and protein profiling of biopsies from non-IBD and IBD patients showed contrasting, but nevertheless, interesting results. We found that despite a tremendous increase in *A20* gene expression in CD patients, A20 protein levels were significantly lower than in non-IBD and UC patients. Thus, focusing further analyses on CD patients, we explored potential factors that could affect A20 post-translationally and negate its function. We found that *TAX1BP1* expression was significantly higher in CD patients, compared to non-IBD controls. *ABIN-1* expression was significantly lower in CD patients, and *IKKβ* expression was unchanged. Decreased *ABIN-1* expression possibly decreased the ability of A20 to bind to its substrates and inhibit inflammation. As well, the decreased *IKKβ* could possibly contribute to degradation of A20, as the

phosphorylated state stabilizes A20 (Hutti et al. 2007). However, this remains to be specifically tested in future studies.

We speculated that bacteria may be affecting A20's progression to or stability as a protein and assessed this by conducting experiments on T-84 cells and *ex-vivo* TI biopsies. To do this, we inoculated two *E. coli* strains, LF82, which is associated with disease recurrence in CD patients, and non-pathogenic, commensal HB101 onto T-84 cells and TI biopsies from non-IBD and CD patients to see the effects of bacteria. Non-pathogenic *E. coli* strains have been shown to stimulate the immune response and increase the expression of *A20* (Zargar et al. 2015). Indeed, treatment of T84 cells with LF82 showed a significant increase in *A20* expression. The expression of other genes was only minimally increased. Remarkably, and similar to our findings in patient biopsies, A20 protein levels were reduced in the LF82-treated group.

Ex vivo inoculation of TI biopsies from CD patients showed a significantly higher increase in *A20* expression, as well, *IKKβ* and *TAX1BP1* expressions increased with all treatments. Surprisingly, there was no change in *ABIN-1* expression after treatment with LF82, and the A20 protein levels were also not increased as much with LF82 treatment.

Treatment of non-IBD biopsies did not cause a change in the gene expression or protein profile. It is, therefore, possible that LF82 acts more in the setting of established inflammation and is not significantly virulent without dysbiosis or disrupted homeostasis.

Our experimental assays show that LF82 caused, in the *in vitro* or *ex vivo* setting, effects that were similar to those seen in TI tissue from patients; this indicates that some

yet undefined, microbial factors could be leading to dysregulation between A20 expression and protein, as well as attenuating the effect of other A20 regulating genes, particularly *ABIN-1*, which could contribute towards lower A20 protein levels.

Our study has several limitations. First, we had to exclude many non-IBD cases on finding inflammation on endoscopy and histology, which decreased our subject numbers. Secondly, as A20 basal expression is usually low (in the absence of local inflammation), it could not be detected in some non-IBD and UC patients. While we have not been able to identify the bacterial factor or mechanism for A20 dysregulation, our findings suggest a novel mechanism for uncontrolled gut inflammation in IBD and that controlling changes in microbes, together with reducing inflammation, could help restore homeostasis in the gut in CD.

Our study has shown two important findings: first, a unique signature profile exists in pediatric CD patients: high *A20* expression and low A20 levels. This reflects a failure of regulation of inflammation in the gut, through A20, likely due to alterations in control and/or stability of the A20 protein. Secondly, we have demonstrated a unique role played by LF82 by its ability to augment *A20* expression, but not its associated genes, which could possibly be a result of lower A20 protein levels in CD patients. It is possible that certain factors induced by inflammation are triggering LF82 to increase *A20* expression.

Our study has demonstrated the importance of A20 dysregulation in pediatric CD and provides important incentives for future studies focusing on regulation of inflammation in pediatric CD as a potential novel therapeutic approach.

CHAPTER 7

Correlation between gut environment and bacterial invasion potential in pediatric inflammatory bowel diseases

7. CORRELATION BETWEEN GUT ENVIRONMENT AND BACTERIAL INVASION POTENTIAL IN PEDIATRIC INFLAMMATORY BOWEL DISEASES

7.1 ABSTRACT:

Introduction: A balanced host-microbial relationship orchestrates the state of homeostasis and immune regulation in the gut. The host-microbial balance is disrupted in IBD. Dysbiosis is a significant feature of IBD. Whether inflammation in IBD leads to the dysbiosis, or whether bacterial dysbiosis initiates inflammation is yet unknown. It is important to understand the role of microbes and the changes in the gut environment in order to better understand the pathogenesis of IBD.

Aim: The hypothesis leading to this study was that the gut luminal environment differs between non-IBD and IBD cases due to the disruption in homeostasis, and that this can alter the virulence capacity of bacteria. The aim of my study was to investigate the relationship between microbial virulence, gut environment, and the gut barrier.

Methods: This study was conducted on 10 non-IBD controls, 9 CD, and 10 UC patients. *In vitro* experiments (gentamicin protection assays) on bacteria isolated from the duodenum and TI of non-IBD and IBD patients were conducted to assess their invasion potential. As well, gentamicin protection assays were conducted on *E. coli* strains

inoculated with intestinal aspirates collected from patients to assess the effects of the gut environment on the bacteria. Metabolomic analysis was conducted on the intestinal aspirates with NMR for quantification of metabolites, in order to identify potential factors that could mediate or explain the observed effects.

Results: Incubation of bacteria with intestinal aspirates from IBD patients, but not non-IBD, significantly altered their *in vitro* invasion potential. NMR was successfully utilized to conduct metabolomic analysis on intestinal aspirates. Lactate and succinate present in the intestinal aspirates correlated positively with induction of bacterial invasion. Thus, intestinal environment in IBD can alter the invasion capacity of bacteria.

Conclusion: The results of this study indicate that alterations in the gut environment in IBD can affect bacterial invasion capacity. Certain metabolites are associated with the increased bacterial invasion and can alter bacterial virulence in IBD. This study provides significant insight into the host-microbe interface that could be used towards future studies designed to elicit host-microbial interactions in IBD.

7.2 Introduction:

Inflammatory Bowel Diseases (IBD), encompassing Crohn disease (CD) and ulcerative colitis (UC) are chronic disorders that prevail in the Canadian pediatric population with a steady temporal increase (Benchimol et al. 2014). Current treatment is targeted towards symptom management and suppression of inflammation and to date no certain etiology or cure have been found. Multiple factors play integrated roles in disease pathogenesis, such as a dysfunctional epithelial barrier (Antoni et al. 2014; Coskun 2014; Weber & Turner 2007; Bruewer et al. 2006), altered microbial composition, and uncontrolled immune responses, but the mechanism of pathogenesis is still unknown. Intestinal homeostasis is disrupted in IBD (Maloy & Powrie 2011), along with an imbalanced innate immune system (Gersemann et al. 2012).

Microbial dysbiosis in IBD has been described by many studies and has been clearly established as a hallmark of IBD (Matsuoka & Kanai 2015). *Peptostreptococcus* and *Eubacteria* species are increased in CD whereas *Bifidobacteria* are reduced (Linskens et al. 2001). Facultative anaerobes are increased in UC. Both UC and CD have reduced *Bacteroidetes* and *Lachnospiraceae. Faecalibacterium prausnitzii* are reduced in CD patients. Another study reported a reduction in Firmicutes and Gammaproteobacteria, and an increase in Enterobacteriaceae in CD and UC patients (Dalal & Chang 2014). Microbiome analysis in stool samples of pediatric UC patients showed an increase in Gammaproteobacteria and reduction in *Clostridia*, along with a reduction in phylospecies in UC patients. Interestingly, patients who failed to respond to intravenous steroid

treatment also had a reduction in the microbial richness at the phylospecies level, compared to those who responded well to therapy (Michail et al. 2012). These disease-related changes in gut microbiota have been associated with increased epithelial permeability, epithelial dysfunction, and increased stimulation of the immune system. Whether bacterial dysbiosis is a cause of inflammation, or whether the ongoing inflammation leads to the dysbiosis is yet unknown, and thus, the role of microbes in IBD needs to be explored in more detail.

It is well established that microbiota impact various aspects of the gut function, especially the epithelial barrier. Gut microbiota play a very important role in maintaining epithelial barrier integrity, as seen in the case of *Lactobacillus plantarum* administration, which leads to an increase in ZO-1 and occludin in tight junctions in biopsies taken from human duodenum (Karczewski et al. 2010). Treatment of Caco-2 cells with *Bifidobacterium infantis* results in increased adhesion of bacteria without damaging the epithelium, and also prevents invasion and adhesion of pathogens such as, *Salmonella typhimurium* and enteropathogenic and enterotoxigenic *Escherichia coli* (Bernet et al. 1993).

Gut metabolism is in a state of homeostasis with a well-balanced relationship between resident microbes and host in the healthy state. This balance is perturbed in IBD, as demonstrated by the case of lower butyrate-producing *Roseburia hominis* in UC. Also, short chain fatty acids, well known for providing energy to colonic epithelial cells, are reduced in UC (Machiels et al. 2013). Gut metabolites reflect the outcomes of cellular processes and host-microbial interactions, and can be used as a tool to understand

disease. Thus, identifying metabolites present in the lumen of the gut and assessing how they differ in the disease and healthy state might help in understanding the complex host-microbial relationship in IBD.

We hypothesized that due to the imbalanced homeostasis in IBD, the gut luminal environment differs between non-IBD and IBD cases, and that this can alter the virulence capacity of bacteria. We tested our hypothesis by conducting *in vitro* experiments on bacteria isolated from the duodenum and TI of non-IBD and IBD patients, and by subjecting *E. coli* strains to intestinal aspirates collected from patients. We found that incubating bacteria with aspirates from the human gut significantly altered their *in vitro* invasion potential and that this was affected by whether they were taken from children with IBD or not. We were able to show that lactate and succinate present in the intestinal aspirates correlated positively with induction of bacterial invasion and have built a rationale for future studies to test the effects of these metabolites on bacterial virulence and invasion.

Our study implies that alterations in the gut environment in IBD can affect bacterial invasion capacity. These findings signify that certain metabolites can alter bacterial virulence in IBD, but this needs to be tested further. Whether it is inflammation or dysbiotic changes in IBD that precipitate alterations in gut metabolite composition, remains to be seen in future prospective or mechanistic studies.

7.3 Methods:

Patients: The patient cohort included in this study was comprised of three groups of pediatric patients; non-IBD, CD, and UC patients who were scheduled to have gastroscopy and colonoscopy at the Stollery Children's Hospital, University of Alberta. Ethics approval from the University of Alberta Research Ethics Board (Study ID Pro00023820) was obtained before recruitment of patients started. The control non-IBD group included children who had gastrointestinal symptoms, such as abdominal pain and diarrhea, and were scheduled for endoscopy. Controls were excluded from the study upon abnormal endoscopic or histological mucosal findings. IBD patients included newly diagnosed and known CD and UC patients as per the Porto criteria (Levine et al. 2014). All patients were directed to maintain a clear fluid diet and ingest Picosalax® (sodium picosulphate with magnesium citrate) the day prior to the procedure and were fasted for at least 6 hours prior to the procedure. The procedures were performed by pediatric gastroenterologists under general anesthesia, administered using propofol by pediatric anesthesiologists.

Collection of intestinal washes: During the procedure, 50 ml of normal saline (NS) injected through the endoscopy working channel were sprayed against the duodenal and terminal ileum (TI) mucosa and aspirates were collected via a suction trap (15-30 ml). Aspirates were cultured overnight on agar plates [LB (Luria-Bertani), MRS (de Man, Rogosa and Sharpe), and McConkey] at 37ºC, under aerobic and microaerophilic

conditions for 24-48 hours. Bacterial colonies grown were isolated and frozen at -80º C in 50% LB-glycerol; the remaining aspirates were frozen in aliquots at -80º C.

Cell culture: Colorectal cell lines, T-84 and Caco-1, were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and 1% penicillin/streptomycin at 37ºC in 5% CO2. Culture medium was changed every other day and cells were passaged every week when confluent. For *in vitro* infection, cells were grown in 24-well plates (seeding density of 1 x 10^6 cells per cm^2) to confluence.

Identification of bacteria isolated from patients: To assess if bacterial invasion potential is altered in IBD, we first identified and then subcultured the bacteria obtained from the cohort to conduct gentamicin protection assays (see below). For this purpose, intestinal aspirates from IBD patients and the control group were plated on LB, MRS, and MacConkey agar plates and cultured for 24-48 hours at 37ºC. Three colonies from each plate were selected based on different morphology for subculture and sequencing, to be used later in the *in vitro* experiments. After culturing in respective broths, the cultures were centrifuged at 10,000g, and the pellet obtained was used for bacterial DNA extraction. DNA was extracted from the bacteria with the DNeasy Blood and Tissue kit (Qiagen, Mississauga, ON, Canada), following the manufacturer's instructions. The 16S rRNA gene of each bacterial isolate was amplified by PCR using universal primers **(Table 7.1)**. PCR products were purified using Qiagen PCR purification kit and analyzed

on 1.5 % agarose gel to confirm that the amplified product is DNA and stored at -80 ºC. The individual isolates were identified with Sanger DNA sequencing (Department of Biological Sciences, University of Alberta, Canada). The DNA Sanger sequencing involves *in vitro* DNA synthesis with a DNA polymerase using chain-terminating dideoxynucleotides. First, a short oligonucleotide primer is annealed on to the DNA strand. The complementary strand synthesis reaction starts with adding each of the four deoxynucleotides (dATP, dCTP, dGTP, dTTP) and the DNA polymerase. Another nucleotide, dideoxynucleotide is also added and acts as the chain-terminator, blocking DNA synthesis. This results in the synthesis of four polynucleotide chains. The DNA is then subjected to polyacrylamide gel electrophoresis which separates the strands to determine their respective lengths.

To assess whether any of the bacteria, particularly *E. coli* strains, carried known virulence factors, FimH, papC, and colibactin were also analyzed using qPCR, and phylotyping was conducted with multiplex PCR, as part of another study (Koleva et al, manuscript in preparation).

Table 7.1: Primers

Target Gene	**Oligonucleotide Sequence (5'-3')**	**Product Size (bp)**
Total Bacteria	F: GAGTTTGATYMTGGCTCAG R: ACTACYNGGGTATTAAKCC	1500

In vitro invasion potential of patient microbes on cell lines: In order to determine if bacterial isolates from non-IBD and IBD patients differed in their capacity to invade epithelial cells, bacterial isolates were cultured overnight in LB broth at 37 ºC under aerobic conditions. Gentamicin protection assays (as described in the section below) were conducted on T84 and Caco-2 cell lines with these bacterial isolates.

Effect of intestinal aspirates on in vitro invasion potential of known E. coli strains: Adherent invasive *E. coli* (AIEC), strain LF82 (Darfeuille-Michaud et al. 2004), non-pathogenic *E. coli* strain HB101, and Enterohemorrhagic *E. coli* (EHEC), strain CL56 (O157: H7) were used for the gentamicin assays, to represent the spectrum of host-microbial relationships (pathobiont, commensal, and pathogen, respectively). The strains were cultured in Luria-Bertani (LB) broth overnight at 37ºC in aerobic conditions before they were used for the inoculation of the cell lines. T-84 and Caco-2 cells were inoculated with either EHEC, LF82, or HB101 at multiplicity of infection (MOI) of 1:20. Each infected well contained 200 µl of aspirates from either non-IBD, CD, or UC patients, or normal saline (as no-aspirate control). The aspirates were syringe filtered through a 0.2 µm filter prior to the experiments to remove debris and bacteria that could have been present in the aspirates. Since the aspirates were mixed with normal saline during collection at the time of endoscopy, we did attempt to normalize the actual volume by quantifying amylase, but it was undetectable in most samples, possibly due to dilution; the same protocol was followed for all cases so aspirate concentrations should be similar between patients. We had used amylase to normalize the intestinal aspirates, as it is

readily detectable in the intestine in the fasting state. Wells containing media only (without bacteria) were used as negative controls.

Prior to infection, the wells were washed thrice with PBS, and antibody free medium (800 µl/well) was added to the wells. The wells were infected with bacteria at an MOI of 1:20 with aspirates/normal saline and incubated for 2 hr at 37°C. After the incubation, 100 µl from each well was plated on agar plates. The wells were washed 3 times with PBS and antibiotic free medium was added (1ml/well). Gentamicin (100µg/ml) was added to each well and incubated for 2 hours. One ml of media without gentamicin was added to Gent – wells; 1 ml with gentamicin to each Gent + well and incubated for 2 hr in 37°C. Suspensions of 100 µl from each well were plated after the 2 hour gentamicin treatment on agar plates to confirm whether bacteria were killed by gentamicin. The wells were washed 3 times with PBS, antibiotic free medium was added (1ml/well), and 100 µl of 1% Triton-X were added to each well to lyse the cells and left at room temp for 15 minutes; cell lysis allows for quantification of invasive bacteria.

After serial dilution, 100 µl from each well was plated on agar plates and placed in aerobic growth conditions for 24hr. The colonies grown on the plates were counted and the number of CFU/well calculated. Bacterial colonies on the plates were counted and the number was multiplied by the dilution factor to reach the number of CFU/well. Given that gentamicin only kills bacteria outside of eukaryotic cells Gentamicin - wells represent both adherent and invasive bacteria and Gent + wells account for only invasive bacteria; the ratios between Gent + and Gent – wells were used to calculate the invasion/adhesion ratio.

Metabolomic analysis of intestinal aspirates: Metabolomic analysis was conducted on intestinal aspirates to determine differences in gut metabolites between non-IBD and IBD cases using nuclear magnetic resonance spectroscopy (NMR). After thawing and centrifuging the intestinal aspirate, 285 µL aliquots of sample were removed and placed in 1.5 mL Eppendorf tubes, followed by the addition of 65 µL of a standard buffer solution [54% D_2O:46%; 1.75mM KH_2PO_4; pH 7.0 v/v containing 5.84 mM DSS (2,2-dimethyl-2-silcepentane-5-sulphonate), 5.84 mM 2-chloropyrimidine-5 carboxylate, and 0.1% NaN_3 in H_2O]. The sample (350 µL) was then transferred to a 3mm SampleJet NMR tube for subsequent spectral analysis. For data collection, 1H-NMR spectra were collected on a 700 MHz Avance III (Bruker) spectrometer equipped with a 5 mm HCN Z-gradient pulsed-field gradient (PFG) cryoprobe. 1H-NMR spectra were acquired at 25°C, using the first transient of the NOESY pre-saturation pulse sequence (noesy1dpr), chosen for its high degree of quantitative accuracy (Saude et al. 2006). All FID's (free induction decays) were zero-filled to 250 K data points. The singlet produced by the DSS methyl groups was used as an internal standard for chemical shift referencing (set to 0 ppm). For quantification, all 1H-NMR spectra were processed and analyzed using an in-house version of the Bayesil automated analysis software package (Ravanbakhsh et al. 2015), using a custom metabolite library. Bayesil software system allows for qualitative and quantitative analysis of an NMR spectrum by automatically and semi-automatically fitting spectral signatures from an internal database to the spectrum. Each spectrum was further analyzed by an NMR spectroscopist to minimize compound misidentification and misquantification. Each metabolite is presented as the percentage of total metabolites.

One metabolite, isopropanol, that represented more than 50% of the total metabolites was excluded from analysis.

Statistical analysis: Data were analyzed using Graph Pad Prism (Graph Pad Software, San Diego, CA, USA). Comparison between the cohort groups was done using ANOVA. Correlation analysis was done using Spearman's correlation coefficient. Statistical significance was determined as $P < 0.05$.

7.4 Results:

Patients: The study was performed on a total of 31 patients. Complete data are available for 29 patients, including 10 non-IBD controls, 9 CD, and 10 UC patients. Two patients were excluded due to technical challenges associated with the NMR analysis of aspirates. The median age of patients was similar between groups. Of the CD cohort, 6 patients were newly diagnosed and 3 were previously diagnosed. All UC patients were previously diagnosed. Patient characteristics, including treatments at time of endoscopy, are described in **Table 7.2**.

Table 7.2: Patient Characteristics

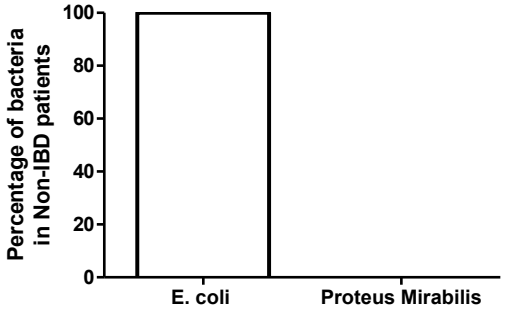
Demographic	***Non-IBD (n=10)***	***CD (n=9)***	***UC (n=10)***
Sex (m/f)	**6/4**	**5/4**	**6/4**
New onset	**NA**	**6**	**0**
Follow-up	**NA**	**3**	**10**
Treatment (# of patients)			
5-aminosalicylic acid	-	2	1
Sulfasalazine	-	2	2
Prednisone	-	3	1
Azathioprine	-	1	2
Methotrexate	-	1	1
Infliximab	-	2	4

IBD, inflammatory bowel diseases; CD, Crohn disease; UC, ulcerative colitis; f, female; m, male.

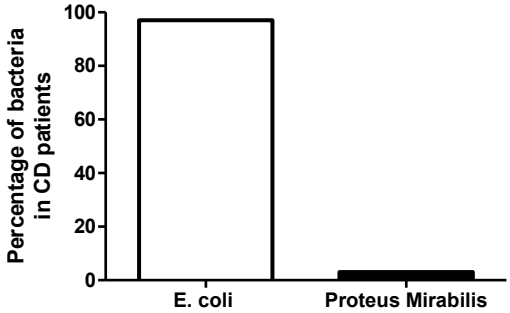
Characterization of bacterial isolates: We plated TI and duodenum aspirates on LB, MRS, and MacConkey agar plates and collected after 48 hr growth bacterial colonies from each plate based on morphology. A total of 100 isolates, from 10 non-IBD, 10 CD, and 10 UC patients were characterized. The bacteria were selected based on differences in morphology on agar plates. The majority of bacterial isolates in all three groups were *E. coli,* as shown in **Fig. 7.1.** This is likely due to the selective culture conditions, which favour growth of *E. coli*, and does not represent the full spectrum of bacteria in the aspirates; given that we needed to culture bacteria for infections we needed to accept this growth bias. There was some variation in the phylotypes and virulence factors of the *E. coli* from the non-IBD and IBD groups, however, the changes were not marked, as shown in **Fig. 7.2**.

Figure 7.1: Majority of aerobic bacterial isolates cultured from terminal ileum aspirates were *E. coli.*

A.



B.



C.

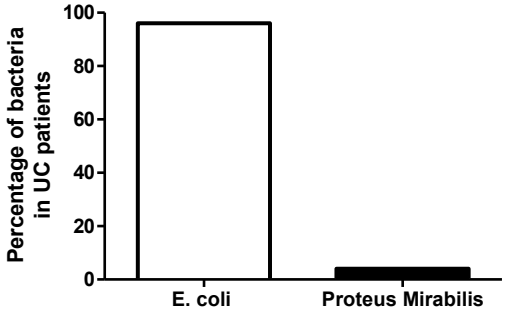
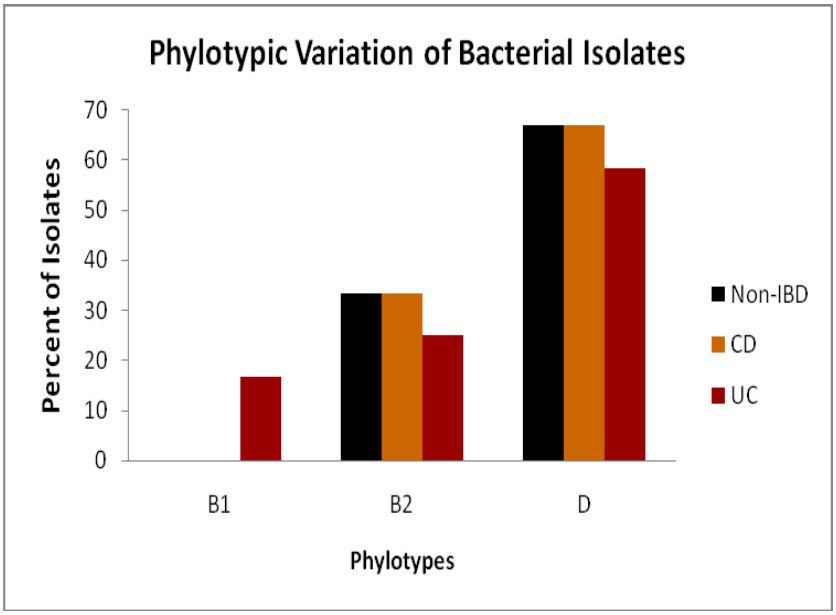


Figure 7.1:

Bacteria obtained by culturing intestinal aspirates and subjected to sequencing showed that the majority of bacteria obtained from all groups were *E. coli,* with a very low percentage of *Proteus Mirabilis.* **A**. Bacteria from non-IBD patients. **B**. Bacteria from CD patients. **C**. Bacteria from UC patients.

Figure 7.2: Phylotypes and virulence factors of bacterial isolates from non-IBD and IBD patients are similar.



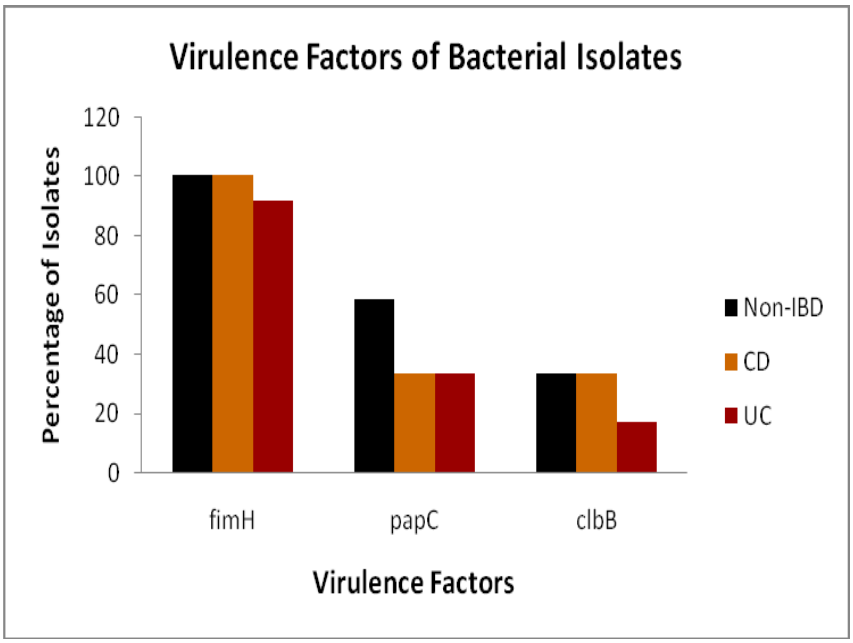


Figure 7.2:

There was only minimal (and not statistically significant) variation amongst phylotypes and virulence factors *fimH, papC*, and *colibactin* in the bacterial isolates of non-IBD, CD, and UC patients (ANOVA, $P>0.05$).

No significant differences in invasion of intestinal epithelial cell lines by bacterial isolates from non-IBD and IBD patients: We compared the invasion of bacteria isolated from non-IBD, CD, and UC patients into T84 and Caco-2 cell lines using the gentamicin protection assay. There was no difference in the invasion capacity of bacteria from the different groups [**Fig. 7.3**: Non-IBD: 983.1 ± 309.5 (mean ± SEM {standard error of the mean}); CD: 1889 ± 1280; UC: 482.7 ± 118.3 CFU/ml; ANOVA: $P>0.05$, N = 14 non-IBD, 8 CD, & 9 UC patients, T84 cell lines]. Similar findings were observed in Caco-2 cells **(Fig. 7.4).** This indicates that even if microbes in patients with IBD are more virulent, this is not apparent after they are removed from the gut environment. It is possible that virulence of bacteria is changed after being subjected to different environmental conditions, such as being removed from the gut and undergoing culture.

Figure 7.3: Invasion potential of intestinal bacteria isolated from TI of non-IBD and IBD patients is similar in T84 cells

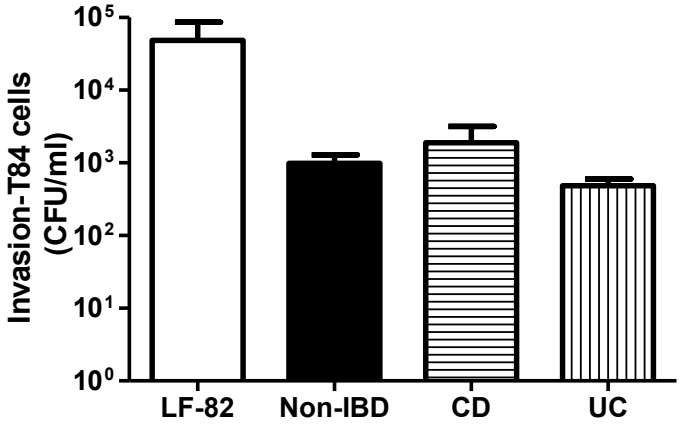


Figure 7.3:

There was no difference in the invasion potential of bacterial isolates from the TI on T-84 cells from Non-IBD, CD and UC patients as compared to AIEC LF-82 strain. However, there was a significant difference ($P<0.05$) between bacteria from patients and LF82, used as a positive control for invasion **(**T84 cells. N= 13; Non-IBD, 9 CD and 10 UC).

Figure 7.4: Invasion potential of intestinal bacteria isolated from non-IBD and IBD patients on Caco-2 cells is similar.

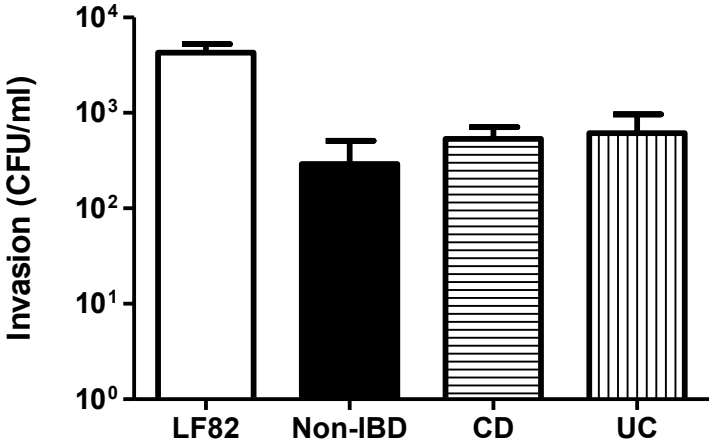


Figure 7.4:

Similar results to T-84 cells were obtained when bacterial isolates were tested on Caco-2 cells. There was no difference in the invasion potential of bacterial isolates from the TI of Non-IBD, CD and UC patients as compared to AIEC LF-82 strain when tested with gentamicin protection assays on Caco-2 cells.

Effects of the intestinal environment on bacterial invasion: Since we did not find a difference in the *in vitro* invasion capacity of bacteria from non-IBD and IBD patients, we hypothesized that the gut environment could alter bacterial virulence. To test this hypothesis, we exposed three well-defined *E. coli* strains with various pathogenic potential to equal volumes of aspirates collected from the duodenum or TI of patients and tested the effects of the aspirates on bacterial invasion using the gentamicin protection assays. The strains we chose were EHEC strain CL56 (O157: H7), *E. coli* strains HB101 and AIEC LF82; these were used to infect T-84 and Caco-2 cells incubated with intestinal aspirates in order to assess the effects of the aspirates on a spectrum of virulence capacities.

Although there was significant variation in the effects of aspirates on bacterial invasion, some striking differences were observed. Incubation with aspirates from the duodenum of UC (but not CD) patients resulted in increased invasion of EHEC compared to those incubated with non-IBD duodenum aspirates [**Fig. 7.5A**: Non-IBD: 920.6 ± 693.6; UC: 4410 ± 1494, CFU/ml; ANOVA: $P<0.05$)], and increased invasion of LF82 when incubated with aspirates from CD patients (but not UC) compared to non-IBD cases (Non-IBD: 206.7 ± 118.8, CD: 5193 ± 917.5 CFU/ml; ANOVA, $P<0.05$). Interestingly, incubation of the bacteria with aspirates from non-IBD patients caused decreased invasion compared to when they were incubated with normal saline.

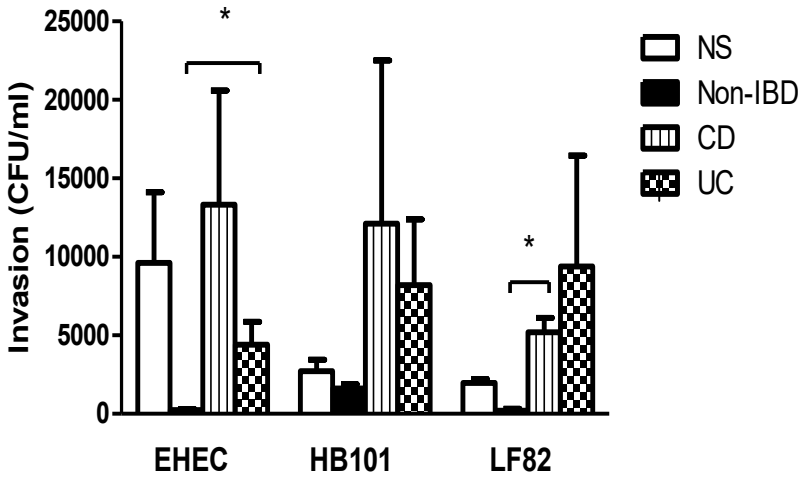
Similar findings were found with incubating bacteria with aspirates from the TI. EHEC invasion in the presence of TI aspirates from UC (but not CD) patients increased significantly compared to those incubated with aspirates from non-IBD cases, [**Fig. 7.5B**:

Non-IBD: 2263 ± 663.9; UC: 72115 ± 54525 CFU/ml; ANOVA: $P<0.05$). The invasion potential of LF82 increased in the presence of TI aspirates from CD (but not UC) patients [**Fig. 7.5B**: Non-IBD 1038 ± 173.2, CD: 4741 ± 2754 CFU/ml; ANOVA: $P< 0.05$, N = 9 non-IBD, CD, & UC patients].

Similar and more striking findings were found in the experiments done with Caco-2 cells (**Fig. 7.6).** Interestingly, invasion potential of both EHEC and LF82 increased when Caco-2 cells were incubated with TI aspirates from CD and UC patients.

Figure 7.5: Invasion potential of bacteria into T84 cells is altered by luminal aspirates from (A) duodenum and (B) TI.

A.



B.

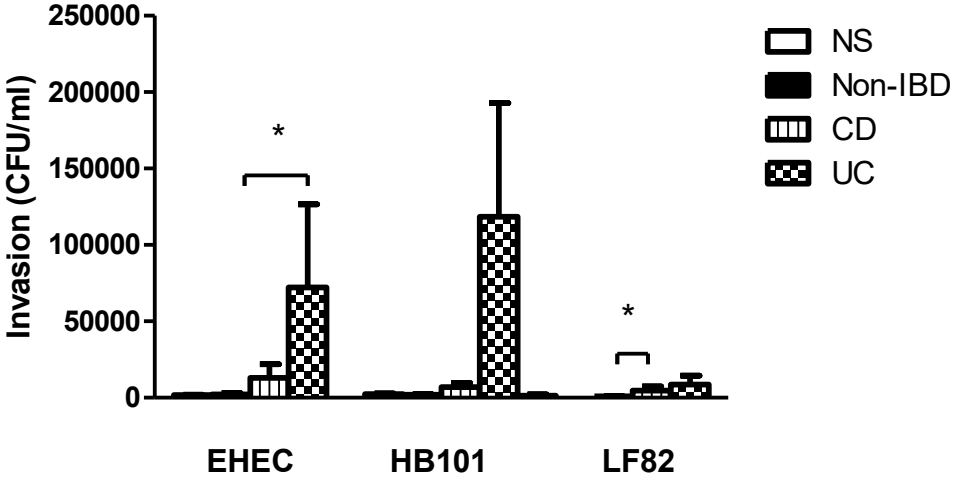


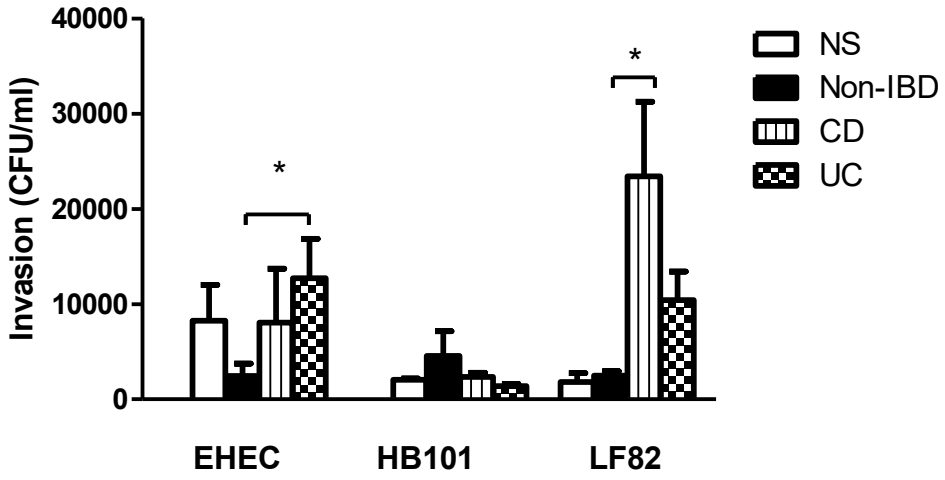
Figure 7.5:

Gentamicin assays on 3 known *E. coli* strains with various pathogenic potential were conducted to test the effects of the intestinal aspirates on bacterial invasion. Incubation with aspirates from the duodenum of UC patients resulted in increased invasion of EHEC as compared to incubated with non-IBD duodenum aspirates (**Fig. 7.5A**: Non-IBD: 920.6 ± 693.6; UC: 4410 ± 1494, CFU/ml; ANOVA: $P<0.05$). Aspirates from CD patients increased invasion of LF82 compared to non-IBD cases (Non-IBD: 206.7 ± 118.8, CD: 5193 ± 917.5; ANOVA: $P<0.05$).

EHEC invasion in the presence of TI aspirates from UC patients increased significantly compared to incubation with aspirates from non-IBD cases (**Fig. 7.5B**: Non-IBD: 2263 ± 663.9; UC: 72115 ± 54525 CFU/ml; ANOVA: $P<0.05$). The invasion potential of LF82 increased in the presence of TI aspirates from CD patients [**Fig. 7.5B**: Non-IBD 1038 ± 173.2, CD: 4741 ± 2754; ANOVA: $P< 0.05$; n = 9 non-IBD, CD, & UC patients].

Figure 7.6: Invasion potential of bacteria is altered by luminal aspirates from (A) duodenum and (B) TI in Caco-2 cells.

A.



B.

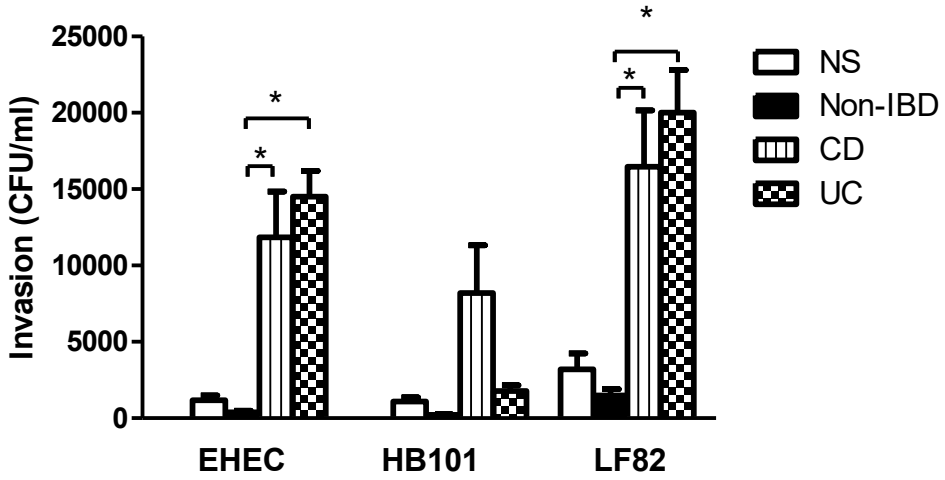


Figure 7.6:

Similar to the findings in T84 cells, incubation of *E. coli* with intestinal aspirates of patients resulted in increased invasion of EHEC and LF82 in both the duodenum and TI, indicating that presence of intestinal aspirates alter the capacity of bacteria to invade cell lines.

Metabolomic Analysis of Intestinal Aspirates: Given the striking effects of intestinal aspirates on bacterial invasion we decided to use an unbiased approach, metabolomics, to identify luminal factors that could either promote bacterial invasion, or at the very least help detect biological processes that would explain this effect. Metabolomic analysis of intestinal aspirates detected, on average, approximately 40 metabolites in each sample (**Fig. 7.7)**. As expected, the quantity of metabolites varied considerably amongst the three study groups and within each group; this did not allow detection of clear statistical changes in patterns between disease groups with the limited number of samples we had and the need for correcting for multiple comparisons.

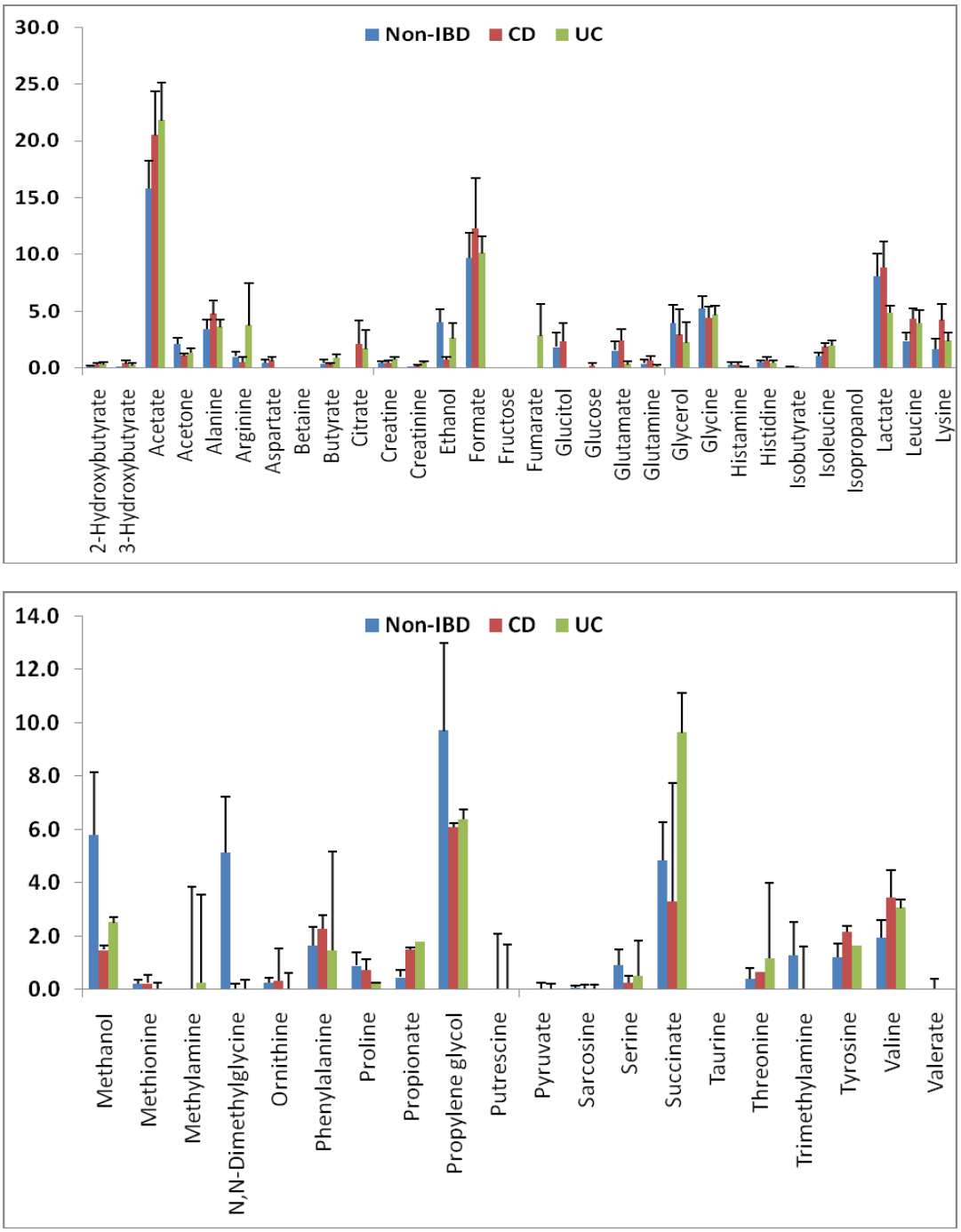
As short chain fatty acids (SCFA) are essential for a healthy gut ecosystem and are involved in multiple key physiologic gut activities, and as reduced SCFA are evidenced in IBD (Huda-Faujan et al. 2010), we focused especially on SCFA analysis. Amongst the SCFAs, acetate was the highest, comprising approximately 15% of all metabolites in both duodenal and TI aspirates and in all patient groups. While there was a clear variation in the quantification of the metabolites, no significant difference was observed amongst the SCFA metabolites of the three patient groups, although formate was present at a lower percentage in CD patients, compared to non-IBD and UC groups in duodenal aspirates.

Of the other metabolites, some regional variation was observed, such as lactate, which seemed to be lower in duodenum (2%) and higher in TI aspirates (8%) in CD patients. Succinate was higher than both non-IBD and UC in the duodenum (11%) and TI aspirates (9%) of CD patients (**Fig. 7.8A & B),** although there was no statistically

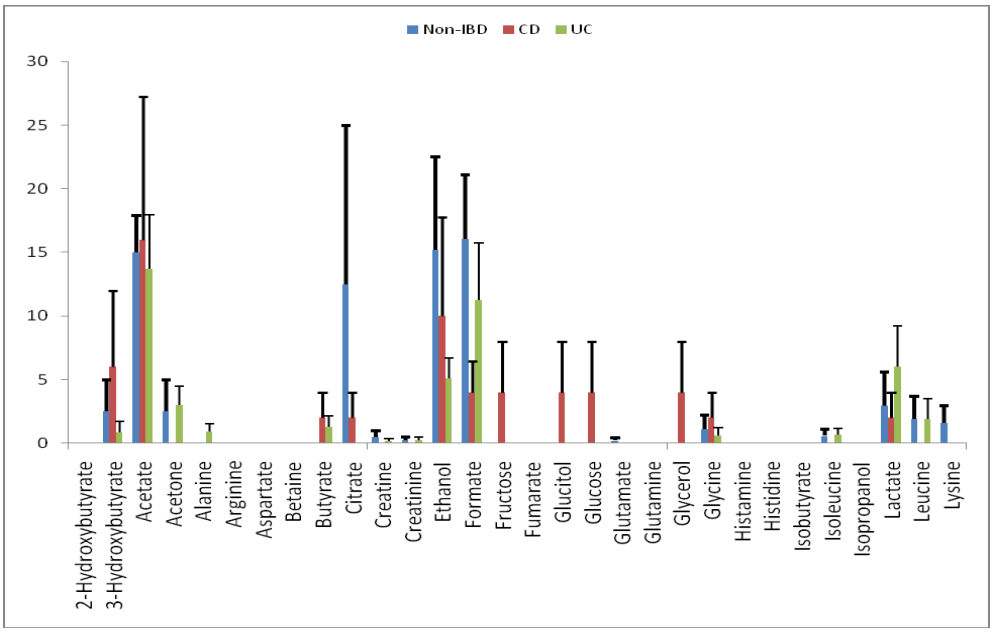
significant difference in absolute quantification of metabolites, possibly due to low number of subjects.

Figure 7.7: Percentage of metabolites in TI (A) and duodenum (B) aspirates

A.



B.


Non-IBD
CD
UC
30
25
20
15
10
5
0
2-Hydroxybutyrate
3-Hydroxybutyrate
Acetate
Acetone
Alanine
Arginine
Aspartate
Betaine
Butyrate
Citrate
Creatine
Creatinine
Ethanol
Formate
Fructose
Fumarate
Glucitol
Glucose
Glutamate
Glutamine
Glycerol
Glycine
Histamine
Histidine
Isobutyrate
Isoleucine
Isopropanol
Lactate
Leucine
Lysine

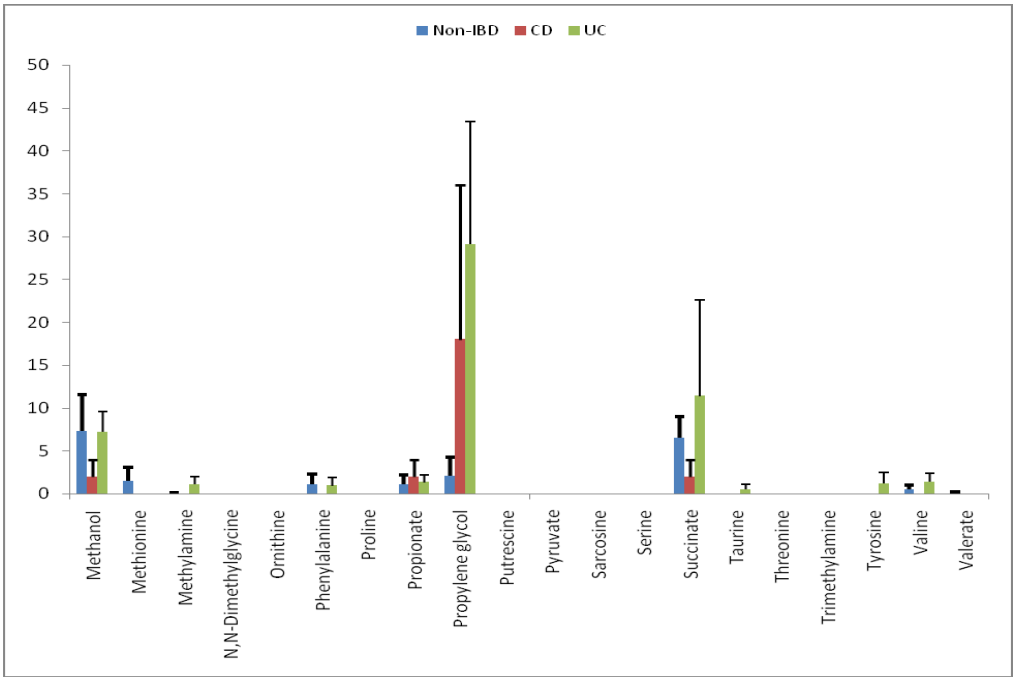

Non-IBD
CD
UC
50
45
40
35
30
25
20
15
10
5
0
Methanol
Methionine
Methylamine
N,N-Dimethylglycine
Ornithine
Phenylalanine
Proline
Propionate
Propylene glycol
Putrescine
Pyruvate
Sarcosine
Serine
Succinate
Taurine
Threonine
Trimethylamine
Tyrosine
Valine
Valerate

Figure 7.7A:

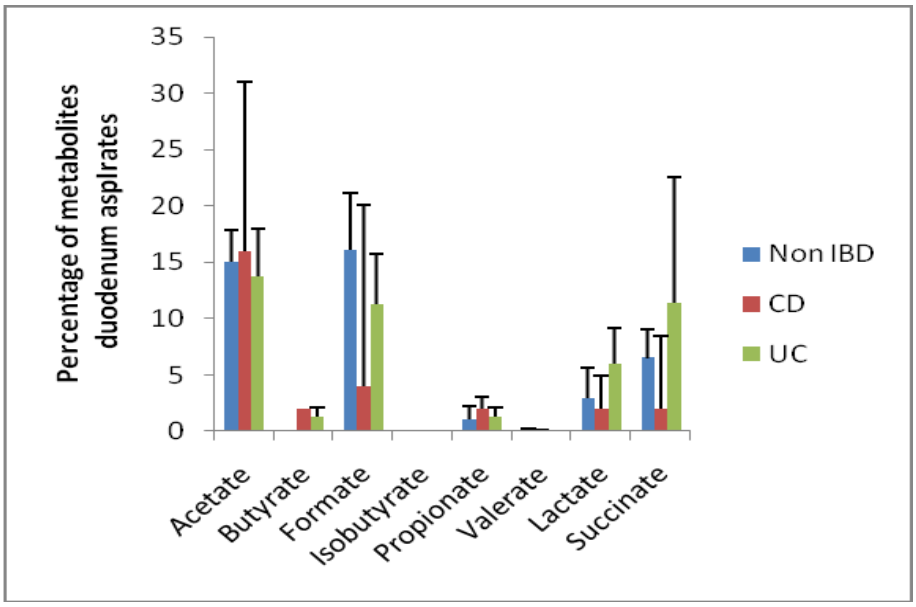
This figure represents the percentage of metabolites present in the TI aspirates of patients as analyzed by NMR. The percentage was obtained by dividing the quantity of each metabolite by the total number of metabolites present in each sample.

Figure 7.7B:

This figure represents the percentage of metabolites present in the duodenal aspirates of patients as analyzed by NMR.

Figure 7.8: Variation in SCFA of Non-IBD and IBD patients in duodenum and TI aspirates

A.



B.

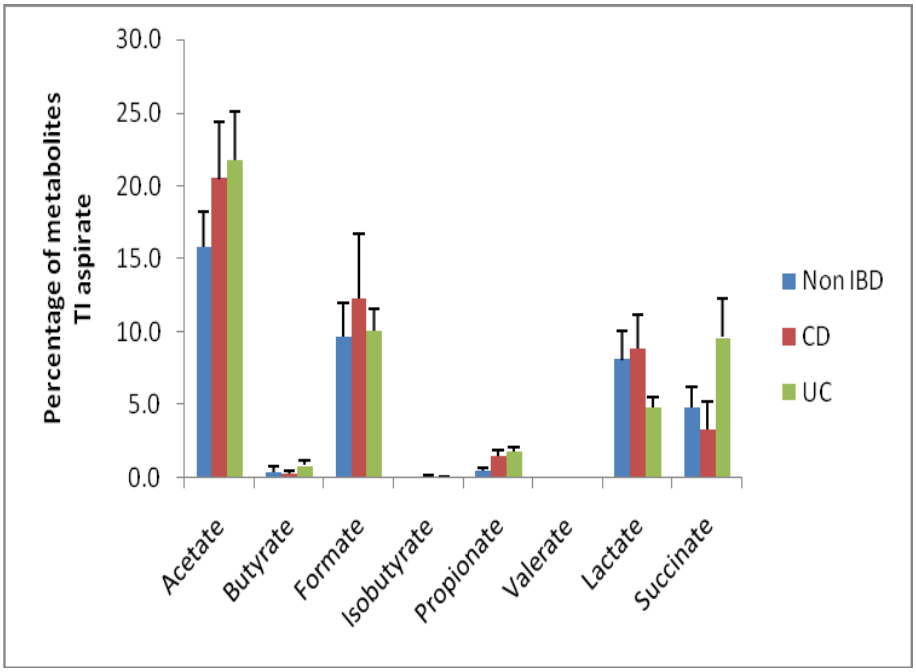


Figure 7.8:

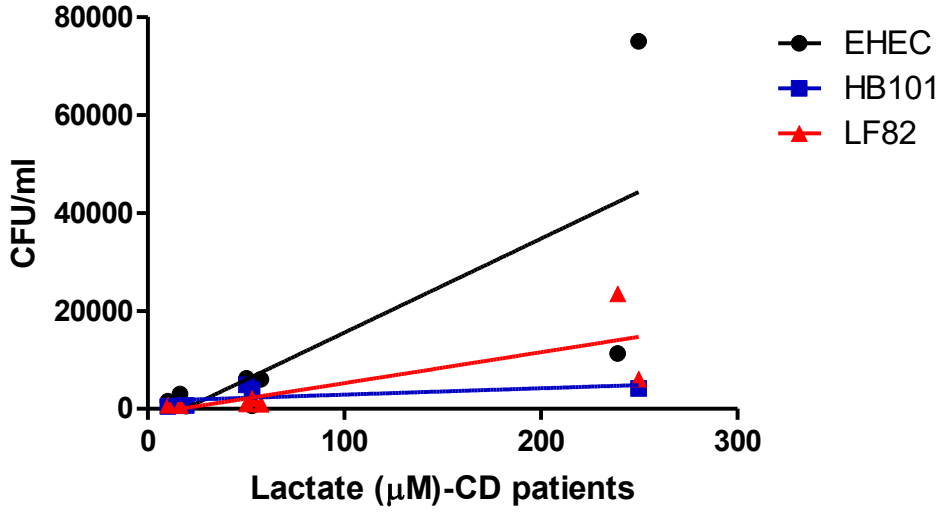
The percentage of SCFA metabolites varied considerably within and amongst the three study groups, although there was no significant difference in the absolute quantification of the metabolites. The percentage of each metabolite was obtained by dividing the absolute number of each metabolite by the total number of metabolites in the sample. **A**. Metabolomic analysis of duodenal aspirates. **B**. Metabolomic analysis of TI aspirates.

E. coli invasion potential correlates positively with succinate and lactate:

As we found that *E. coli* invasion in *in vitro* experiments increased when the bacteria were inoculated with aspirates from IBD patients, we hypothesized that the presence of specific metabolites could affect the invasion potential of *E. coli.* Correlation of metabolites present in the aspirates with *E. coli* invasion in each group showed that LF82 invasion was associated with higher lactate concentrations in TI aspirates from CD patients, and invasion of both LF82 and EHEC was associated with high levels of succinate in UC patients (**Fig. 7.9A:** Spearman's 'r' coefficient: 0.8, $P<0.05$; **Fig. 7.9B:** Spearman's 'r' coefficient: 0.7, $P<0.05$). This implies that the invasion potential of bacteria or the ability of epithelial cells to resist invasion are altered in the presence of lactate and succinate. However, this needs to be proven with additional experiments, such as, incubating bacteria with the metabolites and quantifying their invasion potential. Alternatively, these metabolites may serve as a marker for a biological process associated with bacterial invasion.

Figure 7.9: Positive correlation of *E. coli* invasion potential with succinate and lactate:

A.



B.

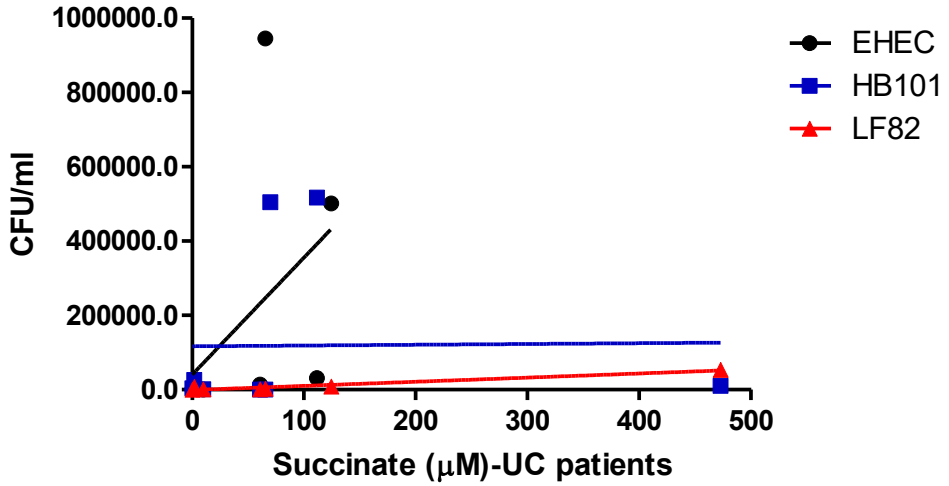


Figure 7.9:

A. LF82 invasion was higher in the presence of higher lactate in TI aspirates from CD patients; Spearman's 'r' coefficient: 0.8, $P<0.05$. **B.** Invasion of LF82 and EHEC was elevated in the presence of high levels of succinate in UC patients; Spearman's 'r' coefficient: 0.7, $P<0.05$.

7.5 Discussion:

The inflammatory trigger of IBD is yet unknown, as is the unclear relationship between the role of microbes and gut barrier. It remains unclear whether the disruption in this relationship is due to host or microbial factors, or both.

The aim of this study was to assess host-microbial interactions in IBD by examining the location where this interface takes place – the gut mucosal surface. We addressed our aim by first analyzing if microbes isolated from intestinal washes from non-IBD and IBD patients differ in their invasive capabilities *in vitro.* There was no difference in the invasion potential of bacteria from non-IBD and IBD groups. This could possibly be due to the fact that once the bacterial isolates are removed from the gut environment and cultured on agar, their virulence changes. This led us to explore the possibility that factors in the gut mucosal interface could be triggering bacterial virulence. To test this we used well-characterized strains of *E. coli* with variable virulent potential. EHEC is a pathogen that is generally non-invasive into intestinal epithelial cells, HB101 is a commensal bacterium without invasive potential, and LF82 is a highly invasive *E. coli* pathobiont, isolated originally from the colon of CD patients (Strober 2011). We found that the rate of invasion of EHEC when incubated with intestinal aspirates from both the duodenum and TI from UC patients increased significantly as compared to that of non-IBD patients. It seemed that aspirates from non-IBD patients had a 'protective effect' against bacterial invasion. The fact that aspirates from the duodenum of UC patients increases EHEC's invasion potential suggests that UC is more of a

systemic disease rather than a regional colorectal disorder (as also observed in **Chapters 3 and 5**).

Incubation of aspirates from CD patients caused an increase in the invasion of the AIEC strain LF82. Thus, our results clearly show that the gut environment and local changes affect how bacteria behave. Indeed, correlation of the metabolites in the intestinal aspirates showed that the invasion rate of *E. coli* correlated positively with high succinate and lactate content in UC and CD patients respectively. Succinate has been found to play a significant role in inflammation, by stimulating dendritic cells (Mills & O'Neill 2014); lactate plays a significant role in regulation of T cells (Haas et al. 2015). Our data shows a correlation in cell invasion and metabolites, however, the role of these metabolites in IBD associated inflammation needs to be further elucidated.

To maintain the synergy that exists between the human host and resident commensals, as well as to control inflammation, a multitude of factors such as diet and products released as a result of bacterial metabolism play an important role. N-butyrate, a metabolite produced by bacterial fermentation of starches inhibits inflammation by macrophages (Chang et al. 2014). A potent inhibitory action on NF-κβ, TNF-α production, and histone deacetylase is facilitated by butyrate, which likely contributes towards gut membrane stability (Plöger et al. 2012). Certain metabolites are altered in IBD, such as, valerate, acetate and propionate that are digestive by-products of bacterial enzymatic actions on carbohydrates. In CD mouse models, metabolite profiling revealed changes in triglycerides and cholesterol (Baur et al. 2011). Short chain fatty acids, which are a major source of energy for colonic epithelial cells are also reduced in UC (Machiels

et al. 2013). In another study, many metabolites identified in stool samples of CD patients were associated with bile acids, fatty acids and amino acid metabolism (Jansson et al, 2009). It is possible that there are certain metabolites/factors present in other parts of the gut, rather than only in the usually inflamed colorectal region, that propagate inflammation through yet unknown mechanisms. This is why I had focused on conducting analysis on the duodenum samples.

Metabolomic analysis is a relatively new tool that is increasingly employed to understand the mechanisms influencing host-microbial interactions and their effects on health. To date, many studies have reported the wide range of techniques used to assess the gut metabolome, such as, mass-spectrometry (Ahmed et al. 2016), NMR (Wissenbach et al. 2016) and Liquid Chromatography (LC)-MS/MS (Yau et al. 2014). The majority of studies on IBD patients have conducted metabolomic analysis on stool samples. One such study on CD patients showed an increase in the metabolites related to tyrosine metabolism, such as, dopaquinone. High amounts of 4-hydroxyphenylacetylglycine were found in a subset of colonic CD patients who had higher *F. prausnitzii* abundance, indicative of a possible correlation between metabolites and microbial composition (Jansson et al. 2009). Another study reported reduced amounts of butyrate, acetate, methylamine and trimethylamine in stool samples of CD and UC patients analyzed with NMR (Marchesi et al. 2007). We have analyzed metabolites in a sample that is in close proximity to the actual disease site, where host cells and microbes intimately interact and perhaps is better reflective of metabolomic changes in IBD than stool samples, especially when studying host-microbe interactions.

Our study does have limitations. Firstly, whether succinate and lactate altered bacterial virulence that resulted in their increased invasion, or if they caused changes in epithelial layer that caused increased invasion of bacteria is yet unknown, and further experiments, such as, gene expression analysis of bacteria after incubation with aspirates need to be done. It is possible that aspirates from IBD patients could have adverse effects on cells in culture; therefore, before conducting the gentamicin assays, cell lines were incubated with aspirates only to see if the cells are destroyed by the aspirates, however, this was not the case. Also, larger sample sizes are required in future studies to validate our findings. It is also important to recognize that we have not proven causality – it is possible that succinate and lactate are markers for another process and that the association with bacterial invasion is indirect. As well, certain factors in non-IBD patients could possibly be inhibitory towards microbial invasion. Still, this novel insight can help direct efforts towards identifying such pathways through metabolic analyses and experimental designs where the effects of these metabolites on cells and bacteria are assessed.

Our study is the first to report the effect of gut environment on the ability of bacteria to invade cells, and we corroborated our results with metabolomic analysis of intestinal aspirates. We have shown for the first time, successful metabolomic analysis of intestinal aspirates using NMR. This is important as analysis of metabolites in the gut might reveal more relevant results than stool samples in IBD. Our findings clearly indicate that the invasion potential of bacteria is increased in the presence of luminal aspirates from IBD patients, and suggest that succinate and lactate are important

metabolites that could be critical drivers (or markers) of microbial virulence in IBD. Future studies need to further confirm the effects of succinate and lactate on bacteria in IBD.

Chapter 8

Discussion, future directions, and significance

8. DISCUSSION, FUTURE DIRECTIONS, AND SIGNIFICANCE.

Chapter 8: Discussion, Future Directions, and Significance.

8.1 Discussion

Pediatric IBD are complicated long term disorders, hallmarked by waxing and waning symptoms, and uncontrolled inflammation (Zhang & Li 2014). The prolonged disease course and the risk of associated complications necessitate in depth probing of potential etiological factors (Langholz 2010). In order to obtain a better understanding of pediatric IBD, and hence improved treatment strategies, it is important to investigate factors associated with pathogenesis and their regulators and to define the complex relationship between involved factors. Through my PhD studies, I addressed the knowledge gaps plaguing the pathogenesis and treatment of pediatric IBD using a translational approach to ensure relevance of my findings. I decided to investigate several critical, interlinked factors associated with IBD pathogenesis, *i.e.*, the gut barrier, microbial virulence, cytokines, and factors that control inflammation. While these disease components address different aspects of pediatric IBD, they are linked in many ways, as demonstrated throughout my thesis and summarized below.

My project revolved around addressing three major aims: analysis of intestinal epithelial gaps, using probe-based confocal microscopy (pCLE); defining immune dysregulation, by studying the mechanistics associated with the anti-inflammatory protein

A20; and assessing host-microbial interactions, utilizing intestinal aspirates for metabolomic analysis and microbial invasion capacity. These three pillars of IBD pathogenesis not only highlight the complex multifactorial nature of IBD but also demonstrate, through my research, the critical links between the intestinal barrier, immune response, and host-microbe interactions. In addition, I also analyzed vascular changes in pediatric IBD with pCLE. My findings support fundamental alterations in all aspects of IBD pathogenesis, showing an increase in epithelial gaps in IBD patients, high capillary flow in the duodenum of UC patients, increased A20 gene expression, but not protein, in CD patients, and finally, alterations in invasion potential of bacteria in the presence of intestinal aspirates, that correlated positively with the succinate and lactate present in the biofluid, quantified using NMR-based metabolomics. In this concluding chapter, the outcomes of my various projects, how they relate to each other, and how they could impact patient care in the future will be discussed.

8.1.1 Increased Epithelial Gap Density in the Non-inflamed Duodenum of Children with Inflammatory Bowel Diseases and its Prognostic Value

The epithelium is an intrinsic part of the intestinal gut barrier, acting as a physical barrier between luminal antigens, microbes, and the ‘heavily armed’ immune tissue beneath. An intact epithelial barrier is critical for curbing inflammatory processes that can be triggered otherwise. Intestinal epithelial cell shedding is a normal phenomenon of gut 'renewal'. Many studies have demonstrated that increased cell shedding occurs in

IBD, outside the parameters of what is considered a physiological phenomenon (Liu et al. 2011), and is related to disease prognosis and predictive of hospitalizations in adults with IBD (Turcotte et al. 2012). As described in **Chapter 3**, we speculated that epithelial gap density is increased in the pediatric population affected by IBD, and confirmed the hypothesis by assessing epithelial gaps in the duodenum of non-IBD, CD, and UC patients, using pCLE, an optical imaging technique that allows microscopic visualization of the mucosal surface during endoscopy (Liu et al. 2011). We have reported, for the first time, increased intestinal epithelial gaps in children affected by IBD as compared to non-IBD controls (Zaidi D, et al. 2016). To our surprise, increased epithelial gaps were present in the duodenum of UC patients (which is considered to be usually uninvolved in UC). Increased epithelial gaps in the absence of inflammation suggests that epithelial gaps could represent a fundamental baseline defect in pediatric IBD. Absence of inflammation was further corroborated by histology and by biopsy cytokine analysis.

This study has provided the baseline for future research of a yet undiscovered area in pediatric IBD pathogenesis, where frank differences (epithelial gaps in this case) are present between non-IBD and IBD cases even in the absence of inflammation, and molecular mechanisms driving this mechanism are largely unknown. An important positive point of our study is the fact that as the duodenum was not inflamed in any of the cases, for further analysis, it will be relatively easy to study molecular mechanisms on biopsy samples, in contrast to other sites where inflammation has already developed, since in such sites, many of the observed findings are likely to be secondary to the inflammation itself.

Epithelial gaps have been reported to be predictive of adverse events in adult CD patients (Turcotte et al. 2012). I wanted to analyze if presence of epithelial gaps in the duodenum of our pediatric IBD cases would show a similar pattern, and completed a follow up study on the patients enrolled in the confocal study for at least 12 months (24 months in some), as described in **Chapter 4.** My aim was to see if higher gap density correlated with/predicted clinical events (hospitalizations, flares), inflammatory parameters (CRP, ESR), disease activity indices (PCDAI/PUCAI), and the need for various treatments.

In contrast to my hypothesis, I did not observe a specific trend, nor a significant difference in the number of clinical events and inflammatory markers amongst patients with normal and high epithelial gap density in either the CD or UC group. This could possibly be due to the fact that very few of our patients actually had flares or required hospitalization; longer follow up may be required to observe differences. My findings did, however, show that infliximab treatment was associated with significantly reduced ESR levels in pediatric CD patients who had high epithelial gap density at the beginning of the follow up period. Reduction in ESR levels did occur in patients with normal gaps who were treated with infliximab too; however, this effect was significantly more marked in patients with high gaps. As a very small number of patients had this effect, it is possible that this conclusion is presumptive. Nevertheless, it is entirely possible that identification of high epithelial gap density using pCLE could potentially be used as a guide to initiate infliximab therapy, as it was associated with good response, presented as a significant drop in ESR levels.

These findings suggest that epithelial gaps reflect an early pathogenic change in pediatric IBD patients. Perhaps since the disease is not present for a long time in children at the time of diagnosis and endoscopy, epithelial gaps are not predictive of clinical events, in contrast to findings in adults; alternatively, our cohort appeared to be mostly well-controlled with very few flares. Long term follow up of these patients into adulthood would provide more information on whether increased epithelial gaps in childhood are predictive of adverse clinical events in adulthood.

8.1.2 Quantitative Analysis of Capillary Flow Rates in the Duodenum of Pediatric Inflammatory Bowel Disease Patients

Encouraged by the novel observation on changes in the epithelium of the non-inflamed duodenum, I focused my attention on other basic biological processes that could be altered in early IBD. Indeed, epithelial gaps were not the only changes found in the unaffected duodenum of our IBD patients. While analyzing the gaps, I observed differences in vascular flow in the confocal images and pursued this finding further, as described in **Chapter 5**. My interest in vascular hemodynamics relates to the critical role it plays in IBD. Inflammation is marked by changes in blood circulation with infiltration of leukocytes (Curtis M Steyers & Miller 2014). Certain cytokines associated with IBD, such as TNF-α, cause increased production of VCAM and MAdCAM-1, which then promote platelet adherence (Cromer et al. 2011) and leukocyte recruitment (Danese 2011). Endothelial cells serve as selective barriers and are disrupted in IBD. Endothelial cells express TLRs, which are responsive to microbes, and thus enable leukocyte

recruitment (Newton & Dixit 2012). In my epithelial gap study, I found increased epithelial gaps in IBD patients. A breach in the epithelium can, as is the case of increased epithelial gaps in the duodenum of our study cases, allow bacterial/antigen entry into the lumen; this can then trigger changes in vascular permeability. This was shown in a dextran sodium sulphate (DSS)-induced colitis mouse model where, as IBD progressed, increased inflammation, IFN-γ and the dysregulated epithelial barrier allowed bacteria to invade and increase microvascular permeability (Oshima et al. 2001b). While vascular alterations are a significant feature of IBD, not much is known about the sequence of events, nor the factors that lead to the full-blown abnormal vascular features of IBD. Since we had the confocal videos available, I decided to explore if there are changes in the vascular flow pattern of IBD patients.

We are the first group to study and successfully use this technique to quantify vascular flow rates at real time in the live duodenum of IBD patients. Several studies have attempted to use confocal endomicroscopy to assess IBD-associated vascular changes. Microvascular changes, such as microvascular tortuosity and fluorescein leakage in the lamina propria, have been assessed with this technique in the absence of frank lesions in UC patients (Buda et al. 2014). Vascular changes have also been successfully assessed for colorectal surveillance (De Palma et al. 2016), as well as for studying vascular changes in sepsis in porcine models (Schmidt et al. 2013). We are the first group to report vascular changes in a non-inflamed site in the duodenum of UC patients. This study has opened up new venues for molecular research related to vascular

associated IBD pathogenesis by demonstrating a quantitative method for assessing flow in live intestinal human tissue.

Thus, using pCLE, my studies have identified two important physical changes that are present in the unaffected duodenum of children with IBD, especially UC: increased epithelial gaps and accelerated vascular flow. It is possible that these two pathological features are closely linked, with epithelial gaps in IBD patients stimulating vascular permeability, leading to increased blood flow and triggering of inflammatory proteins. Testing this hypothesis is outside the scope of my thesis, but is an important point to ponder in future studies, as detailed below. Given that neither of these findings have been described prior to my work in children with IBD, I believe that my work will stimulate further research into fundamental changes in the epithelial and vascular barriers in IBD.

8.1.3 Dysregulation of TNFAIP3 (A20) is Associated with Inflammation in Pediatric Crohn Disease

Once the barrier is breached and vascular and immune responses are stimulated, there is need to regulate these deviations in homeostasis. A major factor hugely impacting current disease management in IBD is failure of controlling inflammation and dysregulation of immune factors. A significant gap in successful IBD management is the lack of identification of factors that regulate inflammation; current therapies are more focused on suppressing inflammation after it has already been initiated. Identification of,

and understanding factors that regulate inflammation can improve IBD management and possibly even allow to prevent disease from developing.

The *A20* gene encodes the zinc finger protein A20, also known as tumor necrosis factor α-induced protein 3 (TNFAIP3). A20 inhibits NF-κβ-induced inflammation. Once gene expression is induced with NF-κβ activation, the protein exerts its deubiquitinating properties on target sites to quench downstream NF-κβ proinflammatory signalling.

The inhibitory action of A20 is dependent on several factors, and I focused on some of them in this study, as they allow A20 to bind to its target site and stabilize it, thus promoting its regulatory effect. A20 interacts with A20-binding inhibitor of NF-κβ activation 1 (ABIN-1) and Tax1 binding protein 1 (TAX1BP-1) to attenuate inflammation, as both of these proteins facilitate A20 to bind to its target sites. ABIN-1 attaches A20 to the IKK/NEMO complex (Mauro et al. 2006) and TAX1BP1 recruits A20 to the IKK complex that leads to breakage of polyubiquitin chains (Verstrepen et al. 2011). Binding of A20 to its target sites is important as it enables the protein to inhibit the NF-κβ cascade. Thus, both ABIN-1 and TAX1BP1 are critical factors essential for A20 to exert its full anti-inflammatory potential. An important factor that could be potentially relevant to TNF-α production and activity is that ABIN-1 inhibits TNF-α-mediated apoptosis (Verstrepen et al. 2009). IKKβ (inhibitor of nuclear factor kappa-B kinase subunit beta), an essential activator of NF-κβ, also significantly affects A20 through phosphorylating, and hence stabilizing it (Hutti et al. 2007).

For the first aim of my thesis, where I had analyzed epithelial gaps in the duodenum of IBD patients **(Chapter 3)**, in order to confirm that the presence of epithelial

gaps did indeed occur in the absence of inflammation, I had analyzed cytokine levels, including TNF-α, as it is an important factor controlling epithelial cell shedding. I wanted to assess if high TNF-α levels could possibly be contributing to elevated epithelial gaps in IBD. I found that neither TNF-α, nor the expression and protein encoded by its counter-regulatory gene *A20* were high in duodenum of IBD patients. On the contrary, TNF-α cytokine levels and *A20* gene expression were high in the TI of CD patients, but A20 protein levels were actually low. This surprising discrepancy between A20 expression and protein interested me and stimulated me to further explore the possible dysregulation of this anti-inflammatory protein in IBD.

In an attempt to define the role of regulatory A20 in pediatric IBD, I hypothesized that failure of this immune-regulatory process could contribute to IBD. Further analysis revealed that the gene expression of *ABIN-1* and *IKKβ* were also low, possibly contributing to low levels of A20 protein due to lack of 'support' and stability provided by ABIN-1 that helps A20 bind to its target sites, as well as due to potential absence of post-translational phosphorylation related to lower expression of IKKβ (a finding that we are currently further pursuing).

I hypothesized that bacteria could possibly be contributing towards this dysregulation between *A20* expression and protein levels and explored this possibility by conducting experiments on T84 cells and *ex-vivo* biopsies. Inoculation of TI biopsies with *E. coli*, strain LF82 increased *A20* expression, but not A20 protein, suggesting its possible role in negatively regulating A20. Thus, dysregulation of the A20 pathway seems to be a contributory factor towards uncontrolled inflammation in pediatric IBD.

My study, however, did have several limitations. I analyzed only a few factors that affect A20 protein. There are several other factors that affect the A20 protein and have not been addressed in this study (mainly due to limited availability of patient tissue), such as paracaspase MALT-1 and reactive oxygen species (Catrysse et al. 2014). My findings strongly suggest that A20 is dysregulated in pediatric CD and analysis of A20-related factors contribute to the current knowledge gap regarding failure of regulation of anti-inflammatory responses in IBD. These results certainly direct future research addressing new potential therapeutic applications through manipulation of A20, as detailed below.

8.1.4 Correlation Between Gut Environment and Bacterial Invasion Potential in Pediatric Inflammatory Bowel Diseases

Microbes play an important role in IBD pathogenesis. After finding interesting results related to the gut barrier and immune response, I wanted to explore how microbes fit in the picture. Secondly, I wanted to see if the altered gut environment, the intestinal aspirates in this case, affects microbes.

There was no difference in the invasion potential of bacteria from non-IBD and IBD patients, as shown by the results of the gentamicin assays. These non-intuitive findings suggested to me that bacteria in IBD patients may not be more virulent because of inherent traits they hold (and indeed, these bacteria did not demonstrate increased virulence factors) but rather are affected by the environment they are exposed to. In order to test the effects of intestinal aspirates on microbial invasion, gentamicin protection assays were conducted on enterohemorrhagic *E. coli* (EHEC), strain CL56 (O157: H7),

non-pathogenic *E. coli*, strain HB101, and adherent-invasive *E. coli* (AIEC), strain LF82 using T-84 and Caco-2 cells after incubating the bacteria with aspirates from the patients. Invasion potential of *E. coli* was significantly increased in the presence of aspirates. In order to explore the constituents of the luminal aspirates that potentially altered bacterial invasion potential, we conducted metabolomic analysis on the intestinal fluid. Correlation analysis showed that bacterial invasion increased in the presence of high levels of lactate in aspirates from CD patients, and succinate in UC patients.

Metabolomics is a an emerging tool in diagnostics and defining disease processes that is increasingly being used to relate cellular processes and their products to disease state, in order to get better understanding of the underlying molecular mechanisms. The term 'metabolomics', described as 'the systemic analysis of the chemical fingerprints of specific cellular processes' (Oliver et al. 1998), is gaining a foothold in development and direction of therapeutics. With the advent of the concept of personalized medicine, metabolomics could be a powerful technique to achieve targeted treatment, as it represents molecular changes related to both intrinsic and extrinsic factors, such as bacteria, host changes, environment, and the relationships between them (Koen et al. 2015).

Gut homeostasis is maintained by a well-orchestrated balance between commensals and the human host. In the diseased state, this homeostatic balance is disrupted. Altered bacterial metabolism reflects changes in microbial composition, diet, and environmental factors. Bacterial actions in the gut produce numerous metabolites, some of which are anti-inflammatory, such as, n-butyrate, a metabolite produced by

bacterial fermentation of starches and other complex carbohydrates that inhibits inflammation by macrophages (Chang et al. 2014). As an example for the relationship between gut microbes and metabolites, alterations in gut microbiome of mice after antibiotic treatment resulted in changes in their urine metabolites (Romick-Rosendale et al. 2009). Dietary changes were also found to alter urine metabolomic profile (Stella et al. 2006). Another study suggested that GC/MS analysis showed that metabolites related to the TCA cycle and certain amino acids were lower in colonic biopsies of UC patients as compared to the non-IBD cohort. Metabolites in the sera of UC patients were different from those of CD and non-IBD patients and was used to differentiate disease phenotype (Ooi et al. 2011). Another study utilized the technique of 1-H NMR spectroscopy for analysis of metabolites present in the urine, sera and plasma of IBD and non-IBD patients, and while the technique was highly powerful in differentiating the metabolites between healthy and IBD patients, there was a difference in the metabolites analyzed in the three biofluids (Schicho et al. 2012). Various animal models, such as, DSS have been used to assess metabolites in colonic tissue, and some metabolites, such as succinate and glutamate have been used to distinguish different stages of colitis (Shiomi et al. 2011).

In **Chapter 7** we analyzed metabolites in intestinal aspirates, a biological specimen that is in close proximity to the actual disease site, and perhaps is better reflective of metabolomic changes in IBD than stool, urine, or sera. We have shown for the first time, successful metabolomic analysis of intestinal aspirates using NMR. Our findings clearly indicate that the invasion potential of bacteria is increased in the presence

of luminal aspirates from IBD patients, and that succinate and lactate are important metabolites that could be reflective of changes in microbial virulence in IBD.

8. 2 Future Directions

The knowledge gaps addressed in my thesis include novel and exciting insights into disease pathogenesis. However, there is still much work to be done to better define the mechanisms involved and to allow for knowledge translation of the results. Amongst the many exciting findings included in this thesis, the most intriguing finding of my research, in my opinion, is the dysregulation of A20. Conditions affecting factors that act as accessories to A20 (ABIN-1 and IKKβ) remain to be explored, as well as the mechanisms by which *E. coli* triggers A20 expression, and the gut barrier. In this section, I have outlined some of the potential future research projects that have stemmed from my work and that my work has provided excellent rational for further pursuit:

Chapter 3: Increased epithelial gap density in the non-inflamed duodenum of children with Inflammatory Bowel Diseases and its Prognostic Value.

My results have shown that epithelial gaps are higher in the duodenum of IBD patients as compared to non-IBD patients, and that there is no association with inflammation in the duodenum. However, several gaps remain to be addressed in order to understand the underlying molecular mechanism and apply the findings for diagnostic and therapeutic purposes. Analysis of human ileum biopsies from IBD patients reported

caspase-1-mediated apoptosis to be a probable mechanism involved in epithelial cell shedding (Liu et al. 2013). However, the cause of increased epithelial gaps in the duodenum is largely unanswered to date and requires further attention.

Cytokine analysis of biopsies of the patients included in the study showed that IL-2 and IL-8 were significantly higher in the duodenum of UC patients. Our results are suggestive that both of these cytokines are related to UC, and early gut barrier disruption in UC can potentially alter immune response. The exact role that these cytokines play in UC remains to be determined. However, consistent with my results, reports from literature do indicate that these cytokines possibly play a strong role in IBD. IL-2 knockout mice developed colitis with features similar to human UC (Sadlack et al. 1993). Other studies reported the presence of IL-8, a chemokine secreted by macrophages and neutrophils, in colonic tissue in IBD patients (Grimm et al. 1996) that was inducible in response to TNF-α and microbes (Hoffmann et al. 2002).

Whether the presence of increased epithelial gaps is due to a delayed turnover of cells, increased cell shedding, or changes in inflammatory markers remains to be seen, and future studies based on animal models of IBD, as well as *in vitro* experiments should be directed towards deciphering potential causes. Experiments analyzing epithelial gaps in IL-2 and IL-8 knockout mice, *in vitro* experiments stimulating cell shedding and subsequent analysis of cytokines on overexpressed/knocked-down IL-2 and IL-8 cell lines will reveal further information. Similar experiments can be conducted *ex-vivo* on organoids cultured from intestinal biopsies of IBD patients.

Surveillance confocal imaging of the same patients over time will help determine if increased epithelial cell shedding is a phenomenon that occurs *a priori* to the appearance of lesions of IBD, or whether the duodenum remains uninflamed over a long period of time.

Chapter 5: Quantitative analysis of capillary flow rates in the duodenum of pediatric inflammatory bowel diseases.

We have successfully quantified capillary flow rates in the duodenum and have shown significant changes in capillary flow in IBD; however, the mechanisms to explain these findings remain to be defined. Vascular changes are a hallmark of IBD, although much needs to be deciphered about this process yet. Marked as an intrinsic part of chronic inflammation, vascular changes have also been associated with IBD (Curtis M. Steyers & Miller 2014). Changes in endothelial cells play a critical role in the vascular pathogenesis associated with IBD. Brachial ultrasound has been used to measure endothelial dysfunction in IBD patients. Evaluated as decreased pulse arterial tonometry (PAT indices) and shear stress reactive hyperemia as compared to non-IBD patients, endothelial dysfunction was not correlated with disease activity in IBD patients (Roifman et al. 2009). Endothelial cells control vascular flow, including leukocyte and platelet infiltration. Endothelial cells prevent platelet aggregation and excessive leukocyte infiltration in the healthy state, and also prevent increased vascular permeability. Vascular blood flow is affected by many factors, such as hypoxia, nitric oxide, endothelin 1, prostacyclin, cell adhesion molecules (Cibor 2016). Angiogenesis is the ultimate step

in IBD-associated vascular changes, and is mediated in large part by leukocytes, platelets, and cytokines (Danese 2011). It is possible that increased vascular flow in the duodenum of IBD patients represent a baseline defect that perpetuates IBD-associated inflammation. However, additional experiments are required to confirm this hypothesis, such as, analysis of adhesion molecules, vascular endothelial growth factors, nitric oxide synthase, and other factors that affect vascular flow in intestinal biopsies in order to determine if these subtle, early changes do occur in remote, seemingly healthy sites in IBD patients. Follow-up pCLE and histology studies on current patients are essential to determine if duodenal inflammation does occur in the long term in these patients. It is possible that increased epithelial gaps in IBD provide a pathway for microbial translocation, altering vascular and immune dynamics. Thus, analyzing both epithelial gaps and vascular flow with pCLE in patients will help determine the possible link between these anomalies.

Therapeutics against inflammatory factors have been shown to positively affect endothelial dysfunction. Use of anti-TNF factors has resulted in a reduction in serum markers of endothelial dysfunction in rheumatoid arthritis patients. Vasodilation has been reported to be improved with anti TNF-α therapy in IBD, psoriasis, and rheumatoid arthritis (Curtis M. Steyers & Miller 2014). However, how anti-TNF measures up to improve endothelium-dependent vasodilation in long-term chronic inflammation, and how it acts in conjunction with other inflammatory markers remains to be seen. Some studies have reported anti TNF-α therapy to be anti-angiogenic and a down regulator of CD40 (Cibor 2016). Furthermore, some biologics used for IBD have potent vascular

effect as well, such as Vedolizumab that inhibits α4-β7 integrins and MAdCAM-1 interaction (Löwenberg & D'Haens 2015). To further validate the benefits of these medications on vascular factors, animal studies are required. IBD mouse models treated with these medications and analysis of vascular factors will reveal more information. Knock-down or over-expression models of important vascular factors and the effects of various medications on cytokines will reveal further information.

Chapter 6: Dysregulation of TNFAIP3 (A20) is Associated with Inflammation in Pediatric Crohn Disease

While this study has demonstrated a probable mechanism related to failure of regulation of inflammation in the gut through A20, my interesting findings have just began to investigate this topic and much remains to be explored. Previous studies have reported that functions of A20 appear to be cell-specific in the intestine. Experiments with A20 are suggestive of preventing apoptosis and stabilizing epithelial barrier in the intestine. Although intestinal epithelial cell deletions in mice did not lead to development of spontaneous inflammation, the mice were more susceptible to experimental colitis, as well as had increased sensitivity to epithelial cell apoptosis stimulated with TNF-α (Vereecke et al. 2010). As well, A20 limits myeloid cells from hyperactivation (Matmati et al. 2011). Mouse models with simultaneous deletions of A20 in both intestinal epithelial and myeloid cells cause significant intestinal inflammation, resulting in severe colitis and ileitis, increased intestinal cell apoptosis, and loss of goblet and Paneth cells (Vereecke et al. 2014).

To further translate my findings towards elucidating the effects of A20 in pediatric IBD patients, a step-wise translational approach needs to be applied. It is entirely possible that A20 expression and protein levels vary amongst intestinal epithelial and dendritic cells, and could be reflective of cell-specific functional variation of A20. Providing inflammatory stimuli, such as LPS, TNF-α, and *E. coli*, strain AIEC LF82 to human dendritic and epithelial cell lines and quantifying cytokines, A20, ABIN-1 expression, and protein levels would further help explore the pathogenesis of A20. Similar experiments on cell lines, in which A20 is knocked down or overexpressed, and on organoids isolated from patients will further help identify the regulatory mechanisms of A20 and ultimately help reach precision medicine goals in patients.

In additional larger cohorts of pediatric IBD patients, protein levels of A20, ABIN-1, TAX1BP1, and IKKβ need to be analyzed to validate my findings. As IKKβ phosphorylates A20 and stabilizes it, it is essential to assess the presence of phosphorylated A20 in IBD patients. Factors that affect A20 and ABIN-1's translation and affect them post translationally need to be explored as well, such as transcriptional factors and microRNAs. Mouse models of A20 knockout mice need to be explored further to see if ABIN-1 is affected in them as well.

Physiologic states of the cell, such as, pH and reactive oxygen species, could all affect the stability of the protein. *In vitro* experiments simulating different cellular states might help elucidate the effects on A20 regulation and stability.

Functional polymorphisms in A20 were found to be predictive of response to anti-TNF therapy in a Danish cohort of IBD patients (Bank et al. 2014). In a larger cohort,

A20 SNPs correlated with anti-TNF therapy (Vereecke et al. 2014). This implies that polymorphisms in *A20* could be used as genetic biomarker to tailor therapy; however, much larger pediatric cohorts need to be analyzed before conclusive results can be reached regarding this 'gene biomarker-therapeutic approach.

Furthermore, I will do immunoflorescence experiments with an antibody against the phosphorylated form of A20 on tissue sections from the TI of non-IBD and IBD patients. The antibody is kindly provided by Dr. Abbott (Case Western Reserve University, Cleveland, USA).

Chapter 7: Correlation between gut metabolites and bacterial invasion potential in pediatric inflammatory bowel diseases

Metabolomic profiling of biofluids has multiple advantages. First, metabolites can be used as a diagnostic marker, and secondly, they can be used as a tool to study pathogenic pathways associated with other factors that influence IBD, such as the gut microbiome, environment, diet, and medications.

We have successfully utilized the technique of NMR to quantify metabolites in intestinal aspirates of patients, which has not been done to date. This is an important step as metabolomic changes at the diseased site are probably the best reflection of the disrupted homeostasis between the host and microbiota. We noticed a trend for various metabolites being high in IBD patients, but this effect was not significantly different from non-IBD patients, probably due to the small sample size and the large number of metabolites identified. Regardless, our study has shown that metabolomic analysis of

intestinal aspirates is possible and does show differences in the metabolite profile. Conducting *in vitro* experiments on cell lines and *ex-vivo* on biopsies/organoids by incubating bacteria with metabolites and assessing their invasion potential, and virulence factors will reveal important information regarding the relationship between bacterial virulence and certain metabolites. Given my results regarding correlation of bacterial invasion potential with succinate and lactate (**Chapter 7**), I will start the analysis by conducting *in vitro* experiments on cell lines with these two metabolites first. Correlation of the metabolomic profile of patients with metagenomics conducted on their gut bacteria might provide information about changes in bacterial metabolism associated with dysbiosis. Future studies should be directed at designing larger cohorts and linking specific metabolite changes with disease type and course. This would help create a metabolomic fingerprint unique to disease phenotype, treatment regimens, and age of patients, which might ultimately contribute towards precision medicine.

8.3 Significance

Given the complexity of IBD, it is critical to address associated knowledge gaps in order to successfully improve patient care. The results of this project have directed future IBD research towards new, yet unexplored venues of IBD pathogenesis. Taken together, the results of my studies show significant findings that are important contributors to the understanding of pediatric IBD, and provide the basis for additional scientific work targeted towards a therapeutic approach. Additional studies based on the

findings of this thesis might ultimately help in improving treatment strategies such as, medications that enhance the gut barrier and reduce epithelial cell shedding, and drugs that target specific proteins and reduce inflammation.

This project has revealed important information about the key factors involved in causing and affecting the course of pediatric IBD. I found that epithelial gaps are increased in non-inflamed areas of IBD, that there is dysregulation in factors controlling inflammation, which can lead to an exaggerated immune response, and I was able to successfully quantify metabolites from intestinal aspirates from patients and associate them with the ability of bacteria to invade epithelial cells.

My results have provided the framework that will be helpful in developing effective therapeutic interventions in IBD patients with additional studies. pCLE seems to be a promising tool to evaluate both epithelial gaps and vascular changes associated with IBD. Indeed, if future studies are able to correlate molecular changes associated with increased epithelial gap density and vascular flow, such findings can lead towards the development of important therapeutic steps addressing these issues. Metabolomic analysis on intestinal aspirates of non-IBD and IBD patients in future studies will help understand the correlation of certain metabolites with IBD. Low levels of A20 protein could be a cause of uncontrolled inflammation in CD. Further exploration of factors affecting the stability and mechanism of action of A20 in IBD patients can help tailor treatment strategies to address inflammation.

8.4 Conclusions

The objectives of my thesis have successfully addressed some of the key fundamental knowledge gaps regarding the pathogenesis of pediatric IBD. Given the complex nature of these disorders, it is critical to understand the basic factors that trigger inflammation, as well as propagate it. My results have provided important information about factors critical in IBD pathogenesis.

I started off my study by analyzing an intrinsic feature of the intestinal epithelium, the epithelial gaps. My study revealed increased epithelial gaps in both CD and UC patients, in the absence of inflammation. This indicates that perhaps increased epithelial gap density is an initial feature of pediatric IBD that ultimately triggers inflammation. The fact that we found pathogenic changes in the seemingly healthy duodenum of UC patients supports this notion. Increased IL-2, IL-8, as well as higher capillary flow rates in the duodenum of UC patients, could possibly be the result of microbes having an unrestricted passage through epithelial gaps to the underlying tissue and triggering inflammation. However, this needs to be validated in future experiments encompassing animal models, cell lines, and patient cohorts. Genomic analysis of the microbiome of the duodenum/TI, and cytokine analysis of *in vitro* and *ex vivo* biopsy models will reveal important information about the linkage between epithelial gaps, microbes and immune changes. Interestingly, TNF-α levels, as well as *A20* gene expression was high in the TI of our patients, but not duodenum. It is entirely possible that epithelial gaps occur as a baseline IBD feature, and later, with the onset of inflammation, cytokines such as TNF-α further accelerate the rate of epithelial shedding

in patients. However, as we did not analyze epithelial gaps in the TI of our patients, this assumption remains inconclusive.

Probing further the high *A20* gene expression in the presence of continued inflammation in the TI of CD patients, I found high TNF-α levels, but low A20 protein levels. The low A20 protein levels were probably associated with low *ABIN-1* that helps A20 to bind to its target sites, as well as unchanged *IKKβ* expression that phosphorylates A20 and hence stabilizes it. Interestingly, *E. coli*, strain LF82 augmented *A20* expression but not protein. Thus bacteria could affect A20, either inhibiting its translation or might affect it post-translationally. Additionally, I found that bacterial invasion potential is altered in the presence of intestinal aspirates in *in vitro* experiments, and lactate and succinate are directly correlated with bacterial invasion potential. Whether the presence of certain metabolites, and gut microbes affect A20 protein or expression of ABIN-1 or IKKβ remains to be seen.

Thus, epithelial gaps, cytokines such as TNF-α, microbes, as well as the gut environmental factors are all inter-linked in the complex network of pediatric IBD pathogenesis. My research has provided fundamental information about the basic factors governing IBD pathogenesis and has laid the foundation for additional research that would be helpful towards understanding pathogenesis and a tailored therapeutic approach.

References:

AAVV, 2001. Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. *Fao & Who*, (October), pp.1–34.

Abraham, C. & Cho, J.H., 2009. Inflammatory bowel disease. *The New England journal of medicine*, 361(21), pp.2066–78.

Abramson, O. et al., 2010. Incidence, prevalence, and time trends of pediatric inflammatory bowel disease in Northern California, 1996 to 2006. *Journal of Pediatrics*, 157.

Adrianto, I. et al., 2012. Association of two independent functional risk haplotypes in TNIP1 with systemic lupus erythematosus. *Arthritis and Rheumatism*, 64(11), pp.3695–3705.

Ahmed, I. et al., 2016. Investigation of faecal volatile organic metabolites as novel diagnostic biomarkers in inflammatory bowel disease. *Alimentary pharmacology & therapeutics*, 43, pp.596–611.

Aird, W.C. et al., 1997. Vascular bed-specific expression of an endothelial cell gene is programmed by the tissue microenvironment. *Journal of Cell Biology*, 138(5), pp.1117–1124.

Alfadhli, a a F., McDonald, J.W.D. & Feagan, B.G., 2005. Methotrexate for induction of remission in refractory Crohn's disease. *Cochrane database of systematic reviews (Online)*, (1), p.CD003459.

Alipour, M. et al., 2016. Mucosal barrier depletion and loss of bacterial diversity are primary abnormalities in paediatric ulcerative colitis. *J Crohns Colitis* 10(4):462-71

Amasheh, S. et al., 2002. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *Journal of cell science*, 115, pp.4969–4976.

Amasheh, S. et al., 2005. Contribution of claudin-5 to barrier properties in tight junctions

of epithelial cells. *Cell and Tissue Research*, 321, pp.89–96.

Amasheh, S. et al., 2009. Na+ absorption defends from paracellular back-leakage by claudin-8 upregulation. *Biochemical and Biophysical Research Communications*, 378, pp.45–50.

Antoni, L. et al., 2014. Intestinal barrier in inflammatory bowel disease. *World Journal of Gastroenterology*, 20(5), pp.1165–1179.

Arsenescu, R. et al., 2008. Signature biomarkers in Crohn's disease: toward a molecular classification. *Mucosal immunology*, 1, pp.399–411.

Atreya, I., Atreya, R. & Neurath, M.F., 2008. NF-kappaB in inflammatory bowel disease. *Journal of internal medicine*, 263(6), pp.591–6.

van Baarlen, P., Kleerebezem, M. & Wells, J.M., 2013. Omics approaches to study host-microbiota interactions. *Current Opinion in Microbiology*, 16(3), pp.270–277.

Balzola, F. et al., 2010. Inducible Foxp3 + regulatory T-cell development by a commensal bacterium of the intestinal microbiota: Commentary. *Inflammatory Bowel Disease Monitor*, 11(2), pp.79–80.

Bank, S. et al., 2014. Associations between functional polymorphisms in the NFkappaB signaling pathway and response to anti-TNF treatment in Danish patients with inflammatory bowel disease. *The pharmacogenomics journal*, 14(6), pp.526–534.

Barron, L. et al., 2010. Cutting edge: mechanisms of IL-2-dependent maintenance of functional regulatory T cells. *Journal of immunology (Baltimore, Md. : 1950)*, 185(11), pp.6426–6430.

Baur, P. et al., 2011. Metabolic Phenotyping of the Crohn ' s Disease-like IBD Etiopathology in the TNF ΔARE / WT Mouse Model. *J Proteome Res*, 10(12), pp.5523–5535.

Benchimol, E.I. et al., 2014. Changing Age Demographics of Inflammatory Bowel

Disease in Ontario, Canada. *Inflammatory Bowel Diseases*, 20(10), pp.1761–1769.

Benchimol, E.I. et al., 2011. Epidemiology of pediatric inflammatory bowel disease: A systematic review of international trends. *Inflammatory Bowel Diseases*, 17(1), pp.423–439.

Benchimol, E.I. et al., 2015. Inflammatory bowel disease in immigrants to Canada and their children: a population-based cohort study. *Am J Gastroenterol*, 110(4), pp.553–563.

Bernet, M.F. et al., 1993. Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. *Applied and Environmental Microbiology*, 59(12), pp.4121–4128.

Bernstein, C.N. et al., 2006. The epidemiology of inflammatory bowel disease in Canada: A population-based study. *American Journal of Gastroenterology*, 101, pp.1559–1568.

Bertiaux-Vandaële, N. et al., 2011. The expression and the cellular distribution of the tight junction proteins are altered in irritable bowel syndrome patients with differences according to the disease subtype. *The American journal of gastroenterology*, 106(12), pp.2165–73.

den Besten, G. et al., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*, 54(9), pp.2325–2340.

Beyaert, R., Heyninck, K. & Van Huffel, S., 2000. A20 and A20-binding proteins as cellular inhibitors of nuclear factor- kB-dependent gene expression and apoptosis. *Biochemical Pharmacology*, 60(8), pp.1143–1151.

Binion, D.G. et al., 1997. Enhanced leukocyte binding by intestinal microvascular endothelial cells in inflammatory bowel disease. *Gastroenterology*, 112(6), pp.1895–907.

Biro, G.P., 1997. Review of physiologic mechanisms in response to anemia. , 156, pp.3–5.

Bjerrum, J.T. et al., 2015. Metabonomics of human fecal extracts characterize ulcerative colitis, Crohn's disease and healthy individuals. *Metabolomics*, 11, pp.122–133.

Bousvaros, A. et al., 2005. A randomized, double-blind trial of Lactobacillus GG versus placebo in addition to standard maintenance therapy for children with Crohn's disease. *Inflammatory bowel diseases*, 11(9), pp.833–839.

Bruewer, M. et al., 2003. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *Journal of immunology (Baltimore, Md. : 1950)*, 171, pp.6164–6172.

Bruewer, M., Samarin, S. & Nusrat, A., 2006. Inflammatory bowel disease and the apical junctional complex. In *Annals of the New York Academy of Sciences*. pp. 242–252.

Bruno, M.E.C. et al., 2015. Correlation of Biomarker Expression in Colonic Mucosa with Disease Phenotype in Crohns Disease and Ulcerative Colitis. *Digestive Diseases and Sciences*, 60(10), pp.2976–2984.

Buda, A. et al., 2014. Confocal laser endomicroscopy for prediction of disease relapse in ulcerative colitis: A pilot study. *Journal of Crohn's and Colitis*, 8(4), pp.304–311.

Buffie, C.G. & Pamer, E.G., 2013. Microbiota-mediated colonization resistance against intestinal pathogens. *Nat Rev Immunol*, 13(11), pp.790–801.

Burisch, J. & Munkholm, P., 2013. Inflammatory bowel disease epidemiology. *Current opinion in gastroenterology*, 29, pp.357–62.

Cakir, M. et al., 2015. Inflammatory bowel disease in Turkish children. *World J Pediatr*, 11(4), pp.331–337.

Camus, M. et al., 2013. Long-term outcome of patients with Crohn's disease who respond to azathioprine. *Clinical gastroenterology and hepatology : the official*

clinical practice journal of the American Gastroenterological Association, 11(4), pp.389–94.

Cario, E. et al., 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *Journal of immunology*, 164, pp.966–972.

Catrysse, L. et al., 2014. A20 in inflammation and autoimmunity. *Trends in Immunology*, 35(1), pp.22–31.

Cerovic, V. et al., 2012. Intestinal CD103− dendritic cells migrate in lymph and prime effector T cells. *Mucosal Immunology*, 6(1), pp.104–113.

Chang, P. V. et al., 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proceedings of the National Academy of Sciences*, 111(6), pp.2247–2252.

Cho, I. & Blaser, M.J., 2012. The human microbiome: at the interface of health and disease. *Nature Reviews Genetics*, 13(4), pp.260–270.

Cibor, D., 2016. Endothelial dysfunction in inflammatory bowel diseases: Pathogenesis, assessment and implications. *World Journal of Gastroenterology*, 22(3), p.1067.

Cobo, E.R. et al., 2015. Colonic MUC2 mucin regulates the expression and antimicrobial activity of β-defensin 2. *Mucosal Immunology*, (April), pp.1–13.

Colman, R.J. & Rubin, D.T., 2014. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. *Journal of Crohn's & colitis*, 8(12), pp.1569–81.

Comito, D. & Romano, C., 2012. Dysbiosis in the pathogenesis of pediatric inflammatory bowel diseases. *International Journal of Inflammation*, 2012.

Coornaert, B. et al., 2008. T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20. *Nature*

immunology, 9(3), pp.263–71.

Coornaert, B., Carpentier, I. & Beyaert, R., 2009. A20: central gatekeeper in inflammation and immunity. *J Biol Chem*, 284(13), pp.8217–8221.

Coskun, M., 2014. Intestinal epithelium in inflammatory bowel disease. *Frontiers in medicine*, 1, p.24.

Côté-Daigneault, J. et al., 2015. Biologics in inflammatory bowel disease: what are the data? *United European gastroenterology journal*, 3(5), pp.419–28.

Cromer, W.E. et al., 2011. Role of the endothelium in inflammatory bowel diseases. *World Journal of Gastroenterology*, 17(5), pp.578–593.

Dalal, S.R. & Chang, E.B., 2014. The microbial basis of inflammatory bowel diseases. *The Journal of Clinical Investigation*, 124(10), pp.4190–6.

Damci, T. et al., 2003. Increased intestinal permeability as a cause of fluctuating postprandial blood glucose levels in Type 1 diabetic patients. *European journal of clinical investigation*, 33, pp.397–401.

Danese, S., 2011. Role of the vascular and lymphatic endothelium in the pathogenesis of inflammatory bowel disease: “brothers in arms”. *Gut*, 60(7), pp.998–1008.

Darfeuille-Michaud, A. et al., 2004. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn’s disease. *Gastroenterology*, 127, pp.412–421.

Day, A.S. & Burgess, L., 2013. Exclusive enteral nutrition and induction of remission of active Crohn’s disease in children. *Expert review of clinical immunology*, 9, pp.375–83; quiz 384.

Day, A.S. & Lopez, R.N., 2015. Exclusive enteral nutrition in children with crohn’s disease. *World Journal of Gastroenterology*, 21(22), pp.6809–6816.

Dicksved, J. et al., 2008. Molecular analysis of the gut microbiota of identical twins with Crohn's disease. *The ISME journal*, 2(7), pp.716–727.

Dixit, V.M. et al., 1990. Tumor necrosis factor-alpha induction of novel gene products in human endothelial cells including a macrophage-specific chemotaxin. *The Journal of biological chemistry*, 265(5), pp.2973–8.

Dotson, J.L. et al., 2010. Extraintestinal manifestations of pediatric inflammatory bowel disease and their relation to disease type and severity. *Journal of pediatric gastroenterology and nutrition*, 51(2), pp.140–145.

Duncan, S.H. et al., 2004. Contribution of acetate to butyrate formation by human faecal bacteria. *The British journal of nutrition*, 91(06), pp.915–923.

Dupont, A. et al., 2015. Antimicrobial peptides and the enteric mucus layer act in concert to protect the intestinal mucosa. *Gut Microbes*, 5(6), pp.761–765.

Eckburg, P.B. et al., 2005. Diversity of the human intestinal microbial flora. *Science (New York, N.Y.)*, 308, pp.1635–1638.

El-Etr, S.H. et al., 2004. Phosphorylation-independent effects of CagA during interaction between Helicobacter pylori and T84 polarized monolayers. *The Journal of infectious diseases*, 190(8), pp.1516–23.

Eszter Mller, K. et al., 2014. Incidence and paris classification of pediatric inflammatory bowel disease. *Gastroenterology Research and Practice*, 2014.

Fernandes. et al., 2016a. Differential expression of key regulators of Toll-like receptors in ulcerative colitis and Crohn's disease: A role for Tollip and peroxisome proliferator-activated receptor gamma? *Clinical and Experimental Immunology*, 183(3), pp.358–368.

Fernandes. et al., 2016b. Differential expression of key regulators of Toll-like receptors in ulcerative colitis and Crohn's disease: A role for Tollip and peroxisome

proliferator-activated receptor gamma? *Clinical and Experimental Immunology*, 183(3), pp.358–368.

Fiehn, O., 2002. Metabolomics - The link between genotypes and phenotypes. *Plant Molecular Biology*, 48(1-2), pp.155–171.

Fish, S.M., Proujansky, R. & Reenstra, W.W., 1999. Synergistic effects of interferon gamma and tumour necrosis factor alpha on T84 cell function. *Gut*, 45(2), pp.191–8.

van der Flier, L.G. & Clevers, H., 2009. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annual review of physiology*, 71, pp.241–260.

Foitzik, T. et al., 1999. Does microcirculation play a role in the pathogenesis of inflammatory bowel diseases? Answers from intravital microscopic studies in animal models. *International Journal of Colorectal Disease*, 14(1), pp.29–34.

Frank, D.N. et al., 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences of the United States of America*, 104, pp.13780–13785.

Franke, A. et al., 2010. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nature genetics*, 42(12), pp.1118–25.

Frolkis, A. et al., 2013. Environment and the inflammatory bowel diseases. *Canadian journal of gastroenterology = Journal canadien de gastroenterologie*, 27(3), pp.e18–24.

Furusawa, Y. et al., 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*, 504(7480), pp.446–50.

Furuse, M. et al., 2002. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *The Journal of cell biology*, 156, pp.1099–1111.

Furuse, M. et al., 1993. Occludin: a novel integral membrane protein localizing at tight junctions. *The Journal of cell biology*, 123, pp.1777–1788.

Le Gall, G. et al., 2011. Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. *Journal of Proteome Research*, 10(9), pp.4208–4218.

Gallo, R.L. & Hooper, L. V., 2012. Epithelial antimicrobial defence of the skin and intestine. *Nature Reviews Immunology*, 12(7), pp.503–516.

Garrod, D. & Chidgey, M., 2008. Desmosome structure, composition and function. *Biochimica et biophysica acta*, 1778, pp.572–587.

Gersemann, M., Wehkamp, J. & Stange, E.F., 2012. Innate immune dysfunction in inflammatory bowel disease. *Journal of Internal Medicine*, 271(5), pp.421–428.

Griffin, L.M. et al., 2015. Improvements in Bone Density and Structure during Anti-TNF-α Therapy in Pediatric Crohn's Disease. *The Journal of clinical endocrinology and metabolism*, 100(7), pp.2630–9.

Grill, J.I. et al., 2015. Intestinal E-cadherin Deficiency Aggravates Dextran Sodium Sulfate-Induced Colitis. *Digestive diseases and sciences*, 60(4), pp.895–902.

Grimm, M.C. et al., 1996. Interleukin 8: cells of origin in inflammatory bowel disease. *Gut*, 38(1), pp.90–98.

Gröschl, B. et al., 2013. Expression of the MAP kinase phosphatase DUSP4 is associated with microsatellite instability in colorectal cancer (CRC) and causes increased cell proliferation. *International Journal of Cancer*, 132(7), pp.1537–1546.

Günzel, D. et al., 2009. Claudin-10 exists in six alternatively spliced isoforms that exhibit distinct localization and function. *Journal of cell science*, 122, pp.1507–1517.

Guo, Y., Lu, N. & Bai, A., 2013. Clinical use and mechanisms of infliximab treatment on inflammatory bowel disease: A recent update. *BioMed Research International*,

2013.

Gupta, P. et al., 2000. Is lactobacillus GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. *Journal of pediatric gastroenterology and nutrition*, 31, pp.453–457.

Haas, R. et al., 2015. Lactate regulates metabolic and proinflammatory circuits in control of T cell migration and effector functions. *PLoS Biology*, 13(7).

Hamer, H.M. et al., 2008. Review article: The role of butyrate on colonic function. *Alimentary Pharmacology and Therapeutics*, 27(2), pp.104–119.

Han, D. et al., 2013. Dendritic Cell Expression of the Signaling Molecule TRAF6 Is Critical for Gut Microbiota-Dependent Immune Tolerance. *Immunity*, 38(6), pp.1211–1222.

Hatoum, O. a, Heidemann, J. & Binion, D.G., 2006. The intestinal microvasculature as a therapeutic target in inflammatory bowel disease. *Annals of the New York Academy of Sciences*, 1072, pp.78–97.

Hatzimanikatis, V. et al., 2004. Metabolic networks: Enzyme function and metabolite structure. *Current Opinion in Structural Biology*, 14, pp.300–306.

Hayden, M.S. & Ghosh, S., 2008. Shared principles in NF-kappaB signaling. *Cell*, 132(3), pp.344–62.

Hoffmann, E. et al., 2002. Multiple control of interleukin-8 gene expression. *Journal of leukocyte biology*, 72(5), pp.847–855.

Hooper, L. V., Littman, D.R. & Macpherson, a. J., 2012. Interactions Between the Microbiota and the Immune System. *Science*, 336(6086), pp.1268–1273.

Huda-Faujan, N. et al., 2010. The impact of the level of the intestinal short chain Fatty acids in inflammatory bowel disease patients versus healthy subjects. *The open biochemistry journal*, 4(Ic), pp.53–58.

Hutti, J.E. et al., 2007. I B Kinase Phosphorylates the K63 Deubiquitinase A20 To Cause Feedback Inhibition of the NF- B Pathway. *Molecular and Cellular Biology*, 27(21), pp.7451–7461.

Hutti, J.E. et al., 2007. IkappaB kinase beta phosphorylates the K63 deubiquitinase A20 to cause feedback inhibition of the NF-kappaB pathway. *Molecular and cellular biology*, 27(21), pp.7451–61.

Ikenouchi, J. et al., 2005. Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells. *The Journal of cell biology*, 171, pp.939–945.

Van Itallie, C., Rahner, C. & Anderson, J.M., 2001. Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. *The Journal of clinical investigation*, 107, pp.1319–1327.

Jansson, J. et al., 2009. Metabolomics reveals metabolic biomarkers of Crohn's disease. *PLoS ONE*, 4(7).

Johansson, M.E. V, Sjövall, H. & Hansson, G.C., 2013. The gastrointestinal mucus system in health and disease. *Nature reviews. Gastroenterology & hepatology*, 10(6), pp.352–61.

John, L.J., Fromm, M. & Schulzke, J.-D., 2011. Epithelial barriers in intestinal inflammation. *Antioxidants & redox signaling*, 15, pp.1255–1270.

Jostins, L. et al., 2012. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*, 491(7422), pp.119–24.

Jurgens, M. et al., 2011. Levels of C-reactive protein are associated with response to infliximab therapy in patients with Crohn's disease. *Clin Gastroenterol Hepatol.* 9(5):421-7

Karczewski, J. et al., 2010. Regulation of human epithelial tight junction proteins by Lactobacillus plantarum in vivo and protective effects on the epithelial barrier.

American journal of physiology. Gastrointestinal and liver physiology, 298, pp.G851–G859.

Katz, J.A., 2004. Treatment of inflammatory bowel disease with corticosteroids. *Gastroenterology Clinics of North America*, 33(2), pp.171–189.

Kau, A.L. et al., 2011. Human nutrition, the gut microbiome and the immune system. *Nature*, 474(7351), pp.327–36.

Kelly, D. et al., 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nature immunology*, 5(1), pp.104–12.

Kelsen, J. et al., 2011. Infliximab induces clonal expansion of T cells in Crohn's disease: A predictor of lymphoma risk? *PLoS ONE*, 6(3).

Kiesslich, R. et al., 2007. Identification of epithelial gaps in human small and large intestine by confocal endomicroscopy. *Gastroenterology*, 133(6), pp.1769–78.

Kiesslich, R. et al., 2012. Local barrier dysfunction identified by confocal laser endomicroscopy predicts relapse in inflammatory bowel disease. *Gut*, 61(8), pp.1146–53.

Kocaman, O. et al., 2006. Endothelial dysfunction in patients with ulcerative colitis. *Inflammatory bowel diseases*, 12(3), pp.166–71.

Koen, N., Du Preez, I. & Loots, D.T., 2015. Metabolomics and Personalized Medicine. *Advances in Protein Chemistry and Structural Biology*.

Kolios, G., Valatas, V. & Ward, S.G., 2004. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology*, 113(4), pp.427–437.

Kolodziej, L.E. et al., 2011. TNFAIP3 maintains intestinal barrier function and supports epithelial cell tight junctions. *PLoS ONE*, 6.

Kopylov, U. et al., 2012. Anti-tumor necrosis factor and postoperative complications in Crohn's disease: Systematic review and meta-analysis. *Inflammatory Bowel Diseases*, 18(12), pp.2404–2413.

Kotlyar, D.S. et al., 2011. A systematic review of factors that contribute to hepatosplenic T-cell lymphoma in patients with inflammatory bowel disease. *Clin Gastroenterol Hepatol*, 9(1), pp.36–41 e1.

Kumar, P. et al., 2009. Molecular mechanisms of endothelial hyperpermeability: implications in inflammation. *Expert reviews in molecular medicine*, 11(June), p.e19.

Kwiterovich, K.A. et al., 1991. Frequency of adverse systemic reactions after fluorescein angiography. Results of a prospective study. *Ophthalmology*, 98(7), pp.1139–1142.

L. Madsen, K., 2012. Enhancement of Epithelial Barrier Function by Probiotics. *Journal of Epithelial Biology and Pharmacology*, 5, pp.55–59.

Lahad, A. & Weiss, B., 2015. Current therapy of pediatric Crohn's disease. *World journal of gastrointestinal pathophysiology*, 6(2), pp.33–42.

Lammert, E., 2014. *Metabolism of Human Diseases* E. Lammert & M. Zeeb, eds., Vienna: Springer Vienna.

Langholz, E., 2010. Current trends in inflammatory bowel disease: the natural history. *Therapeutic advances in gastroenterology*, 3(2), pp.77–86.

Lassen, K.G. et al., 2014. Atg16L1 T300A variant decreases selective autophagy resulting in altered cytokine signaling and decreased antibacterial defense. *Proceedings of the National Academy of Sciences of the United States of America*, 111(21), pp.7741–6.

Lee, T. et al., 2016. Oral versus intravenous iron replacement therapy distinctly alters the gut microbiota and metabolome in patients with IBD. *Gut*, pp.1–9.

Legaki, E. & Gazouli, M., 2016. Influence of environmental factors in the development of inflammatory bowel diseases. *World journal of gastrointestinal pharmacology and therapeutics*, 7(1), pp.112–125.

Leong, R.W., Mitrev, N. & Ko, Y., 2016. Hygiene Hypothesis: Is the Evidence the Same All Over the World? *Digestive diseases (Basel, Switzerland)*, 34(1-2), pp.35–42.

Levine, a & Wine, E., 2013. Effects of enteral nutrition on Crohn's disease: clues to the impact of diet on disease pathogenesis. *Inflamm Bowel Dis*, 19(6), pp.1322–1329.

Levine, A. et al., 2014. ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *Journal of pediatric gastroenterology and nutrition*, 58(6), pp.795–806.

Levine, A. et al., 2011. Pediatric modification of the Montreal classification for inflammatory bowel disease: The Paris classification. *Inflammatory Bowel Diseases*, 17, pp.1314–1321.

Li, M.Y. et al., 2013. Cholera toxin suppresses expression of ubiquitin editing enzyme A20 and enhances transcytosis. *Cellular Physiology and Biochemistry*, 31(4-5), pp.495–504.

Lim, L.G. et al., 2014a. Confocal endomicroscopy identifies loss of local barrier function in the duodenum of patients with Crohn's disease and ulcerative colitis. *Inflammatory bowel diseases*, 20(5), pp.892–900.

Lim, L.G. et al., 2014b. Confocal endomicroscopy identifies loss of local barrier function in the duodenum of patients with Crohn's disease and ulcerative colitis. *Inflammatory bowel diseases*, 20, pp.892–900.

Lin, J., McKenna, B.J. & Appelman, H.D., 2010. Morphologic findings in upper gastrointestinal biopsies of patients with ulcerative colitis: a controlled study. *The American journal of surgical pathology*, 34(11), pp.1672–7.

Linskens, R.K. et al., 2001. The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics. *Scandinavian journal of gastroenterology. Supplement*, pp.29–40.

Liu et al., 2013. Epithelial cell extrusion leads to breaches in the intestinal epithelium. *Inflammatory bowel diseases*, 19(5), pp.912–21.

Liu, J. et al., 2013a. Ubiquitin ligase A20 regulates p53 protein in human colon epithelial cells. *Journal of biomedical science*, 20(1), p.74.

Liu, J. et al., 2013b. Ubiquitin ligase A20 regulates p53 protein in human colon epithelial cells. *Journal of biomedical science*, 20(1), p.74.

Liu, J.J. et al., 2011. Increased epithelial gaps in the small intestines of patients with inflammatory bowel disease: Density matters. *Gastrointestinal Endoscopy*, 73, pp.1174–1180.

Liu, J.J. et al., 2011. Mind the gaps: confocal endomicroscopy showed increased density of small bowel epithelial gaps in inflammatory bowel disease. *Journal of clinical gastroenterology*, 45(3), pp.240–5.

Liu, J.Z. et al., 2015. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nature Genetics*, 47(9), pp.979–989.

Löwenberg, M. & D'Haens, G., 2015. Next-Generation Therapeutics for IBD. *Current gastroenterology reports*, 17(6), p.444.

Ma, A. & Malynn, B.A., 2012. A20: linking a complex regulator of ubiquitylation to immunity and human disease. *Nature reviews. Immunology*, 12(11), pp.774–85.

Mabbott, N.A. et al., 2013. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. *Mucosal immunology*, 6(4), pp.666–77.

MacCallum, A.J. et al., 2006. Campylobacter jejuni-infected human epithelial cell lines

vary in their ability to secrete interleukin-8 compared to in vitro-infected primary human intestinal tissue. *Microbiology*, 152(12), pp.3661–3665.

Machiels, K. et al., 2013. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut*, pp.1–9.

Madara, J.L., 1990. Maintenance of the macromolecular barrier at cell extrusion sites in intestinal epithelium: Physiological rearrangement of tight junctions. *Journal of Membrane Biology*, 116(2), pp.177–184.

Madsen, K.L. et al., 1999. Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. *Inflammatory bowel diseases*, 5, pp.262–270.

Maloy, K.J. & Powrie, F., 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature*, 474, pp.298–306.

Marchesi, J.R. et al., 2007. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. *Journal of Proteome Research*, 6, pp.546–551.

Marchiando, A.M. et al., 2012. NIH Public Access. , 140(4), pp.1208–1218.

Marchiando, A.M. et al., 2011. The epithelial barrier is maintained by in vivo tight junction expansion during pathologic intestinal epithelial shedding. *Gastroenterology*, 140(4), pp.1208–1218.

Martinez-Medina, M. et al., 2009. Biofilm formation as a novel phenotypic feature of adherent-invasive Escherichia coli (AIEC). *BMC microbiology*, 9, p.202.

Martìn-Padura, I. et al., 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *The Journal of cell biology*, 142, pp.117–127.

Matmati, M. et al., 2011. A20 (TNFAIP3) deficiency in myeloid cells triggers erosive

polyarthritis resembling rheumatoid arthritis. *Nature genetics*, 43(9), pp.908–12.

Matsuoka, K. & Kanai, T., 2015. The gut microbiota and inflammatory bowel disease. *Seminars in Immunopathology*, 37(1), pp.47–55.

Mauro, C. et al., 2006. ABIN-1 binds to NEMO/IKK?? and co-operates with A20 in inhibiting NF-??B. *Journal of Biological Chemistry*, 281(27), pp.18482–18488.

McGuckin, M.A. et al., 2009. Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflammatory Bowel Diseases*, 15, pp.100–113.

Mechtler, P. et al., 2015. MicroRNA analysis suggests an additional level of feedback regulation in the NF-kappaB signaling cascade. *Oncotarget*, 6(19), pp.17097–17106.

Michail, S. et al., 2012. Alterations in the gut microbiome of children with severe ulcerative colitis. *Inflammatory Bowel Diseases*, 18(10), pp.1799–1808.

Milatz, S. et al., 2010. Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes. *Biochimica et Biophysica Acta - Biomembranes*, 1798, pp.2048–2057.

Mills, E. & O'Neill, L.A.J., 2014. Succinate: A metabolic signal in inflammation. *Trends in Cell Biology*, 24(5), pp.313–320.

Moayyedi, P. et al., 2015. Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. *Gastroenterology*, 149(1), pp.102–109.e6.

Molodecky, N.A. et al., 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology*, 142, pp.46–54, e42.

Morgan, X.C., Segata, N. & Huttenhower, C., 2013. Biodiversity and functional genomics in the human microbiome. *Trends in Genetics*, 29(1), pp.51–58.

Murch, S.H. et al., 1993. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut*, 34, pp.1705–1709.

Nagalingam, N.A. & Lynch, S. V., 2012. Role of the microbiota in inflammatory bowel diseases. *Inflammatory Bowel Diseases*, 18(5), pp.968–980.

Nenci, A. et al., 2007. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature*, 446(7135), pp.557–561.

Neuman, M.G. & Nanau, R.M., 2012. Inflammatory bowel disease: role of diet, microbiota, life style. *Translational research : the journal of laboratory and clinical medicine*, 160, pp.29–44.

Neurath, M.F., 2014a. Cytokines in inflammatory bowel disease. *Nature reviews. Immunology*, 14(5), pp.329–42.

Neurath, M.F., 2014b. Cytokines in inflammatory bowel disease. *Nature reviews. Immunology*, 14(5), pp.329–42.

Newton, K. & Dixit, V.M., 2012. Signaling in innate immunity and inflammation. *Cold Spring Harbor Perspectives in Biology*, 4(3).

Nicholson, J.K. et al., 2012. Host-Gut Microbiota Metabolic Interactions. *Science*, 336(6086), pp.1262–1267.

Oeckinghaus, A., Hayden, M.S. & Ghosh, S., 2011. Crosstalk in NF-κB signaling pathways. *Nature immunology*, 12(8), pp.695–708.

Oliver, S.G. et al., 1998. Systematic functional analysis of the yeast genome. *Trends in biotechnology*, 16(9), pp.373–378.

Ooi, M. et al., 2011. GC/MS-based profiling of amino acids and TCA cycle-related molecules in ulcerative colitis. *Inflammation Research*, 60(9), pp.831–840.

Oshima, T. et al., 2001a. Interferon-gamma and interleukin-10 reciprocally regulate endothelial junction integrity and barrier function. *Microvascular research*, 61(1), pp.130–143.

Oshima, T. et al., 2001b. Interferon-gamma and interleukin-10 reciprocally regulate endothelial junction integrity and barrier function. *Microvascular research*, 61(1), pp.130–43.

De Palma, G.D. et al., 2016. In vivo assessment of tumour angiogenesis in colorectal cancer: the role of confocal laser endomicroscopy. *Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland*, 18(2), pp.O66–73.

Patil, S. a. & Cross, R.K., 2013. Update in the management of extraintestinal manifestations of inflammatory bowel disease. *Current Gastroenterology Reports*, 15, pp.1–5.

Pelaseyed, T. et al., 2014. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunological Reviews*, 260(1), pp.8–20.

Perez-Moreno, M. & Fuchs, E., 2006. Catenins: Keeping Cells from Getting Their Signals Crossed. *Developmental Cell*, 11, pp.601–612.

Peterson, L.W. & Artis, D., 2014a. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature reviews. Immunology*, 14(3), pp.141–53.

Peterson, L.W. & Artis, D., 2014b. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature reviews. Immunology*, 14(3), pp.141–53.

Plöger, S. et al., 2012. Microbial butyrate and its role for barrier function in the gastrointestinal tract. *Annals of the New York Academy of Sciences*, 1258(1), pp.52–59.

Rakoff-Nahoum, S. et al., 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell*, 118, pp.229–241.

Ravanbakhsh, S. et al., 2015. Accurate, fully-automated NMR spectral profiling for metabolomics. *PLoS ONE*, 10(5).

Reimund, J.M. et al., 1996. Increased production of tumour necrosis factor-alpha interleukin-1 beta, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. *Gut*, 39(5), pp.684–689.

del Rio, M.-L. et al., 2010. Development and functional specialization of CD103+ dendritic cells. *Immunological reviews*, 234(1), pp.268–281.

Rocchi, A. et al., 2012. Inflammatory bowel disease: A Canadian burden of illness review. *Canadian Journal of Gastroenterology*, 26(11), pp.811–817.

Roifman, I. et al., 2009. Evidence of endothelial dysfunction in patients with inflammatory bowel disease. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*, 7(2), pp.175–182.

Romick-Rosendale, L.E. et al., 2009. NMR-basedmetabonomics analysis ofmouse urine and fecal extracts following oral treatment with the broad-spectrum antibiotic enrofloxacin (Baytril). *Magnetic Resonance in Chemistry*, 47(SUPPL. 1).

Rosenfeld, G., Qian, H. & Bressler, B., 2013. The risks of post-operative complications following pre-operative infliximab therapy for Crohn's disease in patients undergoing abdominal surgery: A systematic review and meta-analysis. *Journal of Crohn's and Colitis*, 7(11), pp.868–877.

Ruemmele, F.M., 2010. Pediatric inflammatory bowel diseases: coming of age. *Current opinion in gastroenterology*, 26(4), pp.332–336.

Ruland, J., 2011. Return to homeostasis: downregulation of NF-κB responses. *Nature*

immunology, 12(8), pp.709–14.

Sadlack, B. et al., 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell*, 75(2), pp.253–261.

Sandoo, A. et al., 2010. The endothelium and its role in regulating vascular tone. *The open cardiovascular medicine journal*, 4, pp.302–312.

Sarlos, P. et al., 2014. Genetic update on inflammatory factors in ulcerative colitis: Review of the current literature. *World journal of gastrointestinal pathophysiology*, 5(3), pp.304–21.

Sartor, R.B., 2008. Microbial Influences in Inflammatory Bowel Diseases. *Gastroenterology*, 134, pp.577–594.

Saude, E.J., Slupsky, C.M. & Sykes, B.D., 2006. Optimization of NMR analysis of biological fluids for quantitative accuracy. *Metabolomics*, 2(3), pp.113–123.

Scaldaferri, F. et al., 2009. VEGF-A links angiogenesis and inflammation in inflammatory bowel disease pathogenesis. *Gastroenterology*, 136(2), pp.585–95.e5.

Schicho, R. et al., 2012. Quantitative metabolomic profiling of serum, plasma, and urine by (1)H NMR spectroscopy discriminates between patients with inflammatory bowel disease and healthy individuals. *J Proteome Res*, 11(6), pp.3344–3357.

Schmidt, C. et al., 2013. Confocal laser endomicroscopy reliably detects sepsis-related and treatment-associated changes in intestinal mucosal microcirculation. *British Journal of Anaesthesia*, 111(6), pp.996–1003.

Schumann, M. et al., 2012. Defective tight junctions in refractory celiac disease. *Annals of the New York Academy of Sciences*, 1258(1), pp.43–51.

Sender, R., Fuchs, S. & Milo, R., 2016. Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. *Cell*, 164(3), pp.337–40.

Shan, M. et al., 2013. Mucus Enhances Gut Homeostasis and Oral Tolerance by Delivering Immunoregulatory Signals. *Science*, 447(September), p.-.

Shembade, N. et al., 2011. The kinase IKKα inhibits activation of the transcription factor NF-κB by phosphorylating the regulatory molecule TAX1BP1. *Nature immunology*, 12(9), pp.834–843.

Shiomi, Y. et al., 2011. GCMS-based metabolomic study in mice with colitis induced by dextran sulfate sodium. *Inflammatory Bowel Diseases*, 17(11), pp.2261–2274.

Van der Sluis, M. et al., 2006. Muc2-Deficient Mice Spontaneously Develop Colitis, Indicating That MUC2 Is Critical for Colonic Protection. *Gastroenterology*, 131(1), pp.117–129.

Söderholm, J.D. et al., 2002. Augmented increase in tight junction permeability by luminal stimuli in the non-inflamed ileum of Crohn's disease. *Gut*, 50, pp.307–313.

Solem, C.A. et al., 2005. Correlation of C-reactive protein with clinical, endoscopic, histologic, and radiographic activity in inflammatory bowel disease. *Inflammatory bowel diseases*, 11, pp.707–712.

Soo, J. et al., 2013. Use of exclusive enteral nutrition is just as effective as corticosteroids in newly diagnosed pediatric Crohn's disease. *Digestive Diseases and Sciences*, 58(12), pp.3584–3591.

Sosinsky, G.E. & Nicholson, B.J., 2005. Structural organization of gap junction channels. *Biochimica et biophysica acta*, 1711, pp.99–125.

Stella, C. et al., 2006. Susceptibility of human metabolic phenotypes to dietary modulation. *Journal of Proteome Research*, 5(10), pp.2780–2788.

Steyers, C.M. & Miller, F.J., 2014. Endothelial dysfunction in chronic inflammatory diseases. *International Journal of Molecular Sciences*, 15(7), pp.11324–11349.

Steyers, C.M. & Miller, F.J., 2014. Endothelial dysfunction in chronic inflammatory

diseases. *International journal of molecular sciences*, 15(7), pp.11324–49.

Strober, W., 2011. Adherent-invasive E. coli in Crohn disease: Bacterial "agent provocateur." *Journal of Clinical Investigation*, 121(3), pp.841–844.

Suenaert, P. et al., 2002. Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. *American Journal of Gastroenterology*, 97, pp.2000–2004.

Tamura, A. et al., 2011. Loss of claudin-15, but not claudin-2, causes Na+ deficiency and glucose malabsorption in mouse small intestine. *Gastroenterology*, 140, pp.913–923.

Teshima, C.W., Dieleman, L.A. & Meddings, J.B., 2012. Abnormal intestinal permeability in Crohn's disease pathogenesis. *Annals of the New York Academy of Sciences*, 1258, pp.159–165.

Tobin, J.M. et al., 2001. Upper gastrointestinal mucosal disease in pediatric Crohn disease and ulcerative colitis: a blinded, controlled study. *Journal of pediatric gastroenterology and nutrition*, 32(4), pp.443–8.

Tong, M. et al., 2014. Reprograming of gut microbiome energy metabolism by the FUT2 Crohn's disease risk polymorphism. *The ISME journal*, 8(11), pp.1–14.

Tsukita, S., Furuse, M. & Itoh, M., 2001. Multifunctional strands in tight junctions. *Nature reviews. Molecular cell biology*, 2, pp.285–293.

Turcotte, J.-F. et al., 2012. Increased epithelial gaps in the small intestine are predictive of hospitalization and surgery in patients with inflammatory bowel disease. *Clinical and translational gastroenterology*, 3(7), p.e19.

Turnbaugh, P.J. et al., 2007. The human microbiome project. *Nature*, 449(7164), pp.804–10.

Turner, D. et al., 2007. Development, validation, and evaluation of a pediatric ulcerative colitis activity index: a prospective multicenter study. *Gastroenterology*, 133,

pp.423–432.

Turner, D. et al., 2012. Mathematical weighting of the pediatric Crohn's disease activity index (PCDAI) and comparison with its other short versions. *Inflammatory Bowel Diseases*, 18, pp.55–62.

Turner, J.R. et al., 2011. Redistribution of the tight junction protein ZO-1 during physiologic shedding of mouse intestinal epithelial cells. *American journal of physiology Cell physiology*.

Ungaro, R. et al., 2012. Protein-losing enteropathy in ulcerative colitis. *Case Reports in Gastroenterology*, 6(1), pp.177–182.

Vaishnava, S. et al., 2008. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proceedings of the National Academy of Sciences*, 105(52), pp.20858–20863.

Veereman-Wauters, G. et al., 2012. Risk of infection and prevention in pediatric patients with IBD: ESPGHAN IBD Porto Group commentary. *Journal of Pediatric Gastroenterology and Nutrition*, 54(6), pp.830–837.

Venkatesh, K. et al., 2009. Feasibility of confocal endomicroscopy in the diagnosis of pediatric gastrointestinal disorders. *World Journal of Gastroenterology*, 15(18), pp.2214–2219.

Vereecke, L. et al., 2014. A20 controls intestinal homeostasis through cell-specific activities. *Nature communications*, 5, p.5103.

Vereecke, L. et al., 2010. Enterocyte-specific A20 deficiency sensitizes to tumor necrosis factor-induced toxicity and experimental colitis. *The Journal of experimental medicine*, 207(7), pp.1513–1523.

Vereecke, L., Beyaert, R. & van Loo, G., 2009. The ubiquitin-editing enzyme A20 (TNFAIP3) is a central regulator of immunopathology. *Trends in Immunology*,

30(8), pp.383–391.

Verstrepen, L. et al., 2009. ABINs: A20 binding inhibitors of NF-??B and apoptosis signaling. *Biochemical Pharmacology*, 78(2), pp.105–114.

Verstrepen, L. et al., 2011. TAX1BP1, a ubiquitin-binding adaptor protein in innate immunity and beyond. *Trends in Biochemical Sciences*, 36(7).

Wagner, S. et al., 2008. Ubiquitin binding mediates the NF-kappaB inhibitory potential of ABIN proteins. *Oncogene*, 27(26), pp.3739–45.

Watson, A.J.M. et al., 2005. Epithelial barrier function in vivo is sustained despite gaps in epithelial layers. *Gastroenterology*, 129(3), pp.902–12.

Watson, A.J.M. et al., 2009. Mechanisms of epithelial cell shedding in the Mammalian intestine and maintenance of barrier function. *Annals of the New York Academy of Sciences*, 1165, pp.135–42.

Watson, A.J.M. & Hughes, K.R., 2012. TNF-??-induced intestinal epithelial cell shedding: Implications for intestinal barrier function. *Annals of the New York Academy of Sciences*, 1258, pp.1–8.

Weber, C.R. & Turner, J.R., 2007. Inflammatory bowel disease: is it really just another break in the wall? *Gut*, 56, pp.6–8.

Weiss, G. & Gasche, C., 2010. Pathogenesis and treatment of anemia in inflammatory bowel disease. *Haematologica*, 95(2), pp.175–178.

Wenzel, U.A. et al., 2014. Spontaneous colitis in Muc2-deficient mice reflects clinical and cellular features of active ulcerative colitis. *PLoS ONE*, 9(6).

Wertz, I.E. et al., 2004. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kB signalling. *Nature*, 430(7000), pp.694–699.

Wertz, I.E. et al., 2015. Phosphorylation and linear ubiquitin direct A20 inhibition of

inflammation. *Nature*, 528(7582), pp.1–6.

Wine, E., 2014. Should we be treating the bugs instead of cytokines and T cells? *Digestive Diseases*, 32(4), pp.403–409.

Wissenbach, D.K. et al., 2016. Optimization of metabolomics of defined in vitro gut microbial ecosystems. *International Journal of Medical Microbiology*.

Yannuzzi, L.A. et al., 1986. Fluorescein angiography complication survey. *Ophthalmology*, 93(5), pp.611–617.

Yau, Y.Y. et al., 2014. Bimodal plasma metabolomics strategy identifies novel inflammatory metabolites in inflammatory bowel diseases. *Discovery medicine*, 18(98), pp.113–24.

Zaidi D, et al. 2016 Increased Epithelial Gap Density in the Non-Inflamed Duodenum of Children with Inflammatory Bowel Diseases. *Journal of Pediatr. Gastroenterol & Nutrn.* (in press)

Zargar, A. et al., 2015. Bacterial secretions of nonpathogenic Escherichia coli elicit inflammatory pathways: A closer investigation of interkingdom signaling. *mBio*, 6(2).

Zhang, Y.-Z. & Li, Y.-Y., 2014. Inflammatory bowel disease: pathogenesis. *World journal of gastroenterology : WJG*, 20, pp.91–9.