Linköping University Medical Dissertation

No. 1627

**Bacterial epithelial interaction in intestinal
inflammation**

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About the cover:

The front cover displays a simplified model of the ileum, representing villus epithelium and follicle-associated epithelium, including epithelial cells, M cells, Paneth cells and goblet cells that are important in the present study. Schematic representation of examples of commensal and pathogenic bacteria that are present in the ileal lumen.

During the course of the research time underlying the thesis Lina Yakymenko Alkaissi was enrolled in *Forum GIMIICum*, the national research school in gastrointestinal inflammation, which is based at Linköping University, Sweden.

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ISBN: 978-91-7685-278-1

ISSN: 0345-0082

Printed by LIU-Tryck, Sweden, 2018

*Success is most often achieved by those
who don't know that failure is inevitable*

Coco Chanel
(1883-1971)

French designer, businesswoman, inspiration to women everywhere

To Hammoudi, Sabrina and Oliver

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ABSTRACT

The intestine is constantly exposed to bacteria, invading viruses and ingested food. The intestinal barrier serves as a gate preventing passage of harmful components and at the same time maintaining absorption of nutrients and water. There are over 300 different bacteria species in the human gastrointestinal tract (GI) comprising over 10 times as many cells as the human body. These bacteria are both of commensal and pathogenic strains in which commensal bacteria and antimicrobial peptides have an important role of controlling the intestinal colonization. The intestinal flora is sampled by the membranous cells (M cells) that are present in the follicle associated epithelium (FAE). Antigens encounter immune cells found in Peyer's patches located in the distal ileum with FAE overlaying them. Due to environmental factors, genetic predisposition, immune dysregulation or dysbiosis the balance can be shifted which, in turn, will lead to the defect in the barrier function, leading to the development of disorders such as Crohn's disease (CD). CD is a chronic inflammation in the GI tract, often originating in the distal ileum in FAE and associated with an increased number of adherent invasive strains of bacteria. Specifically adherent invasive *E.coli* (AIEC) that have been isolated from the ileum and colon of CD patients.

The aim of the present thesis was to study bacterial epithelial interaction during inflammation in *in vivo*, *ex vivo* and *in vitro* models.

In the first project, we found that that *Faecalibacterium prausnitzii* (FP) possess anti-inflammatory properties in the ileum of an *in vivo* DSS induced colitis mouse model.

In the second project, we discovered that infliximab, known to have anti-inflammatory effects by binding soluble TNF and blocking TNF receptors, reduces bacterial transcytosis across colonic biopsies of CD patients and decreases transcytosis and internalization in cell monolayers *in vitro*. Moreover, we demonstrated that HM427 bacteria, isolated from colonic mucosa of CD patients, use lipid raft formations to penetrate the barrier under the influence of TNF in an *in vitro* model.

In project three, we demonstrated that LF82 bacteria, which are an adherent invasive strain of *E.coli*, isolated from the ileum of CD patients, exploit FAE of CD patients and non-IBD control patients to penetrate the barrier via the CEACAM6 receptor and long polar fimbriae. We further demonstrated that there is an increased expression of CEACAM6 receptor in the FAE of CD patients, which leads to increased transcytosis of LF82 compared to non-IBD control group.

In project four, our results suggested that human α -defensin 5 significantly decreases the passage of LF82 bacteria in an *in vitro* and *ex vivo* models. Moreover, we demonstrated that CD patients have a lower expression of human α -defensin 5 in the crypts compared to the non-IBD control patients.

Taken together, our findings have given a novel insight into the etiology of CD and into the mechanisms involved in bacterial-epithelial interaction in CD.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Tarmen är ett viktigt organ i kroppen som har ett flertal funktioner. En av dem är att ta upp vatten och näring från maten vi äter och transportera det till blodet. För att skydda kroppen mot bakterier och andra skadliga ämnen i tarmlumen, finns en barriär mellan insidan och utsidan av kroppen. Det finns både goda och onda bakterier i tarmen och de goda har en viktig roll genom att försvara tarmen mot att skadliga bakterier tar sig igenom barriären. Många system arbetar tillsammans för att utföra detta försvar och detta inkluderar immunförsvaret. En försvarsmekanism tarmen har är produktionen av anti-mikrobiella peptider som kallas för defensiner och dessa kan döda skadliga bakterier. Det finns ansamlingar av immunceller, s.k. Peyerska plack som är lokaliserade i vissa delar av tunntarmen. Det epitel som täcker de Peyerska placken kallas för follikelassocierat epitel (FAE), och det är specialiserat för att transportera innehåll från lumen till underliggande vävnad. FAE är viktigt för inducering av kroppens skyddande immunförsvaret men fungerar också som ingångsväg för farliga bakterier vilket kan orsaka infektion. Kroppens försvarsreaktion blir då att dra till sig fler immunceller från blodet som släpper ut speciella försvarssubstanter för att rensa bort bakterierna och reparera eventuella skador på kroppens celler och detta kan leda till en inflammation i tarmen som i sin tur leder till vävnadsskada vilket underlättar för ytterligare bakterier att ta sig igenom. En av de tarmsjukdomar som uppstår vid denna typ av inflammation är Crohns sjukdom, en kronisk inflammation i tarmkanalen som vanligtvis startar i FAE regionen. Symptom som kan uppstå är magsmärta, diarré, blod i avföringen, feber och viktnedgång. I Sverige påverkas ungefär 0.5-1% av populationen av denna sjukdom. Orsaken är inte helt känd men många saker kan leda till dess utveckling som till exempel diet, rökning, genetisk känslighet och nedsatt immunsvaret. Man har dessutom hittat ökad förekomst av vissa typer av skadliga bakterier i tarmen hos patienter med Crohns sjukdom. Två typer av dessa skadliga bakterier är *E.coli* LF82 och HM427. Det finns dessutom studier som visar att patienter med Crohns sjukdom inte producerar tillräckligt med antimikrobiella substanser och dessa patienter visar en mera aggressiv immunrespons, vilket förvärrar inflammationen och leder till att bakterier tar sig igenom barriären mycket lättare.

Övergripande syftet med avhandlingen var att studera interaktion mellan bakterier och epitelet, i möss med tarminflammation, patienter med Crohns sjukdom och i en cellodlingsmodell av FAE.

I första projektet undersökte vi hur den goda bakterien *Faecalibacterium prausnitzii* påverkar barriärfunktion och tarminflammation hos möss med en tjocktarmsinflammation. Resultaten visade att bakterien kunde reducera tarminflammationen hos mössen och förbättra barriären. I det andra projektet studerade vi transporten av den skadliga bakterien HM427 hos patienter med Crohns sjukdom både före och efter en behandling som reducerar den immunologiska signalsubstansen TNF. Patienter med behandling visade en lägre transport av HM427 bakterier genom tarmvävnaden. I det tredje projektet visade vi att den skadliga bakterien LF82 passerar till högre grad genom FAE jämfört med vanligt villusepitel, passagen var högre hos patienter med Crohns sjukdom jämfört med kontroller, och att LF82 är beroende av receptorn CEACAM6 och små trådliknande utskott s.k. fimbrier för att passera. I fjärde projektet studerade vi hur antimikrobiella substanser (defensiner) påverkar transporten av LF82 genom FAE. Resultaten visade att defensiner har en positiv effekt på kroppen genom att reducera passagen av LF82 bakterier genom FAE regionen hos patienter med Crohns sjukdom.

Sammantaget bidrar våra studier med djupare kunskap om mekanismer för utvecklingen av Crohns sjukdom, och kan ge förslag till nya möjliga behandlingar.

LIST OF PAPERS

PAPER I

Anders H Carlsson, **Olena Yakymenko**, Isabelle Olivier, Fathima Håkansson, Emily Postma,

Åsa V Keita & Johan D Söderholm

Faecalibacterium prausnitzii supernatant improves intestinal barrier function in mice DSS colitis

Scandinavian Journal of Gastroenterology, 2013, Aug; 48:10, 1136-1144

PAPER II

Olena Yakymenko, Ida Schoultz, Elisabeth Gullberg, Magnus Ström, Sven Almer, Conny

Wallon, Arthur Wang, Åsa V. Keita, Barry J. Campbell, Derek M. McKay, Johan D. Söderholm

Infliximab restores colonic barrier to adherent-invasive E.coli in Crohn's disease via the effects on epithelial lipid rafts

Scandinavian Journal of Gastroenterology, 2018, Apr; **In press**

PAPER III

Åsa V Keita, **Olena Yakymenko**, Elin B Holm, Stéphanie DS Heil, Benoit Chassaing,

Derek M McKay, and Johan D. Söderholm

Enhanced E. coli LF82 translocation through follicle-associated epithelium in patients

with Crohn's disease is dependent on long polar fimbriae and CEACAM6. In manuscript

PAPER IV

O. Yakymenko, J. D. Söderholm, Å. V. Keita

Human α -defensin 5 alters uptake of adherent invasive E. coli LF82 in vitro and in human

follicle-associated epithelium of patients with Crohn's disease and in control subjects. In manuscript

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ABBREVIATIONS

AIEC – Adherent invasive *E.coli*

ATG16L1 – Autophagy-related protein 16-1

C57BL/6 mice – C57 black 6 mice

CD – Crohn's disease

CEACAM – Carcinoembryonic antigen related cell adhesion molecule

DLG5 – Disks large homolog 5

DSS – Dextran sodium sulfate

FAE – Follicle associated epithelium

FBS – Fetal bovine serum

FP – *Faecalibacterium prausnitzii*

GALT – Gut associated lymphoid tissue

GFP – Green fluorescent protein

GI tract – Gastrointestinal tract

IBD – Inflammatory bowel disease

IgA – Immunoglobulin A

JAM – Junctional adhesion molecules

LPF – Long polar fimbriae

M cells – Microfold cells

MDR1 – Multi drug resistance gene

MLCK – Myosin light chain kinase

mβcd – Methyl-β-cyclodextrine

NLR – Nod-like receptor

NOD – Nucleotide binding oligomerization domain

PPs – Peyer's patches

TER – Transepithelial resistance

TJ – Tight junctions

TLR – Toll-like receptor

TNF – Tumor necrosis factor

UC – Ulcerative colitis

VE – Villus epithelium

XPB1 – X-box binding protein

ZO-1 – Zonula occludens protein – 1

Introduction

The human gastrointestinal tract (GI tract) is composed of several different organs and can be divided into the upper and the lower GI tract. The upper GI tract includes the mouth, esophagus, stomach, duodenum, jejunum and ileum. The lower GI tract includes the colon, rectum and anus.

The GI tract has a complex innate and adaptive mucosal immune system that has the ability to monitor the luminal content and differentiate between commensal microbiota and food antigens versus the invasion of toxic pathogens [1].

The small intestine is an important component of the GI tract that permits the breakdown and absorption of the nutrient needed for the body to properly function. It also plays a protective role as a part of the barrier function. The small intestine consists of a complex network of blood vessels, nerves, and muscle tissue that all work together to achieve the best results. The small intestine is divided into several layers: the serosa, muscularis, submucosa and mucosa. The serosa layer is the outside layer consisting of mesothelium and epithelium. The muscularis layer as its name might suggest consists of two muscle layers with the nerves located between them. The submucosa consists of a layer containing blood vessels, lymphatics and nerves. The mucosa layer is the innermost layer with its main function being absorption and therefore the mucosa is covered with villi that are protruding into the lumen. The crypt layer of the bowel is the area where cell renewal and proliferation take place. The cells move from the crypt to the villi and change into either enterocytes, goblet cells, Paneth cells or enteroendocrine cells [2].

Mucus serves as a layer over the intestinal epithelium [3], the mucus in the ileum fills the space between the villi, it is not attached to the epithelium and its structure allows particles even large bacteria to penetrate [4]. The mucus layer serves as a diffusion barrier with a high concentration of antimicrobial products [3, 5].

Colon is an organ of maintenance that retrieves water, electrolytes and energy [6]. Energy is derived from partially absorbed sugar and from dietary fiber that got transferred from the small intestine into the colon and was metabolized by bacteria into short chain fatty acids that can then be easily absorbed [7, 8]. Large numbers of bacteria reside in the lumen of the colon, their interaction under normal conditions is either mutually beneficial or beneficial to one but not

harmful to the other. However, once an imbalance occurs in these relationships it could lead to the development of different diseases, for instance, inflammatory bowel disease [9, 10].

Barrier function

The intestinal mucosa as mentioned above is constantly exposed to bacteria, ingested food, and invading viruses [11]. Clearly, the body requires absorbing nutrition from the ingested food. That is why the barrier function is a very important component of the immune system that prevents harmful components from getting into the blood stream and at the same time it has the ability to select the necessary components to be absorbed. The intestinal barrier function includes physical diffusion barriers, regulated physiological barriers as well as immunological barriers [11].

The intestinal barrier consists of several layers and the first line of defense is the lumen [12]. In this layer bacteria and antigens are degraded by gastric acid, pancreatic, and biliary juices that are secreted into the lumen. The commensal bacteria play a very important role in controlling the colonization of the lumen by pathogenic bacteria, by producing antimicrobial substances such as bacteriocins and they affect the pH of the lumen content and compete for nutrients needed for the survival of pathogens [13].

The microclimate is another line of defense. It includes an unstirred water layer, the glycocalyx and the mucus layer that secretes IgA, all these components prevent the adhesion of bacteria to the epithelium binding sites thus making the epithelial-bacterial interaction impossible.

The following layer of defense is the epithelium layer. Epithelial cells are tightly connected to each other via junctional complexes, they respond to the harmful stimuli by secreting chloride. Epithelial cells also have the ability to secrete antimicrobial peptides; the functions of these are to kill bacteria, to attract monocytes and to potentiate macrophage opsonization.

The final layer is lamina propria. It includes the entire nervous and endocrine systems and it consists of innate and acquired immunity cells that secrete immunoglobulins and cytokines. The regeneration of epithelial cells can also be considered as one of the intestinal barrier functions [14]. The epithelium that lines the mucosal surface of the ileum is either regular epithelium otherwise called villus epithelium (VE) or follicle-associated epithelium (FAE) [15].

Peyer's patches

The gut-associated lymphoid tissue (GALT) is made of isolated aggregated lymphoid follicles that form Peyer's patches (PPs). PPs are considered to be the immune sensors of the intestine [16].

PPs are divided into three main domains: the follicle area, the interfollicular area, and the follicle-associated epithelium [15]. The follicular and interfollicular areas consist of the PPs lymphoid follicles with a germinal center (GC) that contains proliferating B-lymphocytes, follicular dendritic cells (FDCs), and macrophages. The follicle is surrounded by the subepithelial dome that mainly consists of B-cells, T-cells, macrophages, and dendritic cells [16].

Studies have shown that the proportion of enterocytes vs. M cells in the FAE can be influenced by the microbiota in the lumen. For instance when mice are transferred from germ free to regular housing the number of M cells in the FAE increases [17]. Moreover, the presence of pathogenic bacteria such as *Streptococcus pneumoniae* or *Salmonella typhimurium* can also increase the number of M cells in the FAE [18, 19].

Transport and uptake across the intestinal barrier

The uptake of water, nutrition, antigens and even bacteria and viruses is an important function of the intestinal barrier; it is of great importance to the uptake control. There exist different routes through which the uptake can take place. Lipid soluble and small hydrophilic compounds can pass through the epithelial cells via passive diffusion into the lipid bilayers or via small hydrophilic aqueous pores. Larger molecules up to 600DA *in vivo* and up to 10 k DA *in vitro* in cell line models can find their way through the tight junctions [20] intracellular spaces as well as intracellular spaces using the paracellular route [21, 22].

Endocytosis

Medium sized particles such as bacterial products and proteins cannot pass through the cell membrane or via the paracellular path, and in this case these particles are absorbed through endocytosis [23]. Endocytosis is a process of active transport of particles by the cell, during which the cell infolds the plasma membrane which is followed by a vesicle formation. After endocytosis the particles that have been taken up are transported and degraded via the lysosomal pathway of the enterocytes [24]. Endocytosis can occur in different ways depending on the nature of the particles that are being taken up. The first route is via clathrinmediated endocytosis which takes place via specific receptors. Mostly growth factors from breast milk, immunoglobulins and viruses

are taken up via this route [25, 26, 27]. During this type of endocytosis epithelial cells express apical membrane receptors and internalize receptor specific molecules [27].

Endocytosis via lipid rafts

Endocytosis via the lipid rafts/caveolae mechanism is another endocytosis type. This entry way is mostly used by enterotoxins and viruses. This process of endocytosis via the lipid rafts formation involves a flask shaped invagination of cholesterol containing microdomains in the plasma membrane that, in turn, contains a coat protein, caveolin [28].

Macropinocytosis

Macropinocytosis is a non-specific process through which large amounts of extracellular fluid, dissolved molecules, large particles, viruses, bacteria and cell fragments that have gone through apoptosis get internalized. The macropinocytosis process starts by the invagination of the cell membrane, during which the single cell lamellipodia gets bent and gives rise to circular ruffles that are then released into the cytoplasm as a vesicle (macropinosome) [29]. It has previously been shown that the macropinocytosis process is increased in response to antigen stimulation in M cells and enterocytes [30, 31].

Phagocytosis

Larger bacteria and particles can be taken up via phagocytosis or absorptive endocytosis [31]. The process of phagocytosis involves the binding of molecules to the cell membrane via receptors. This process is triggered by the soluble substances secreted by invading bacteria [32]. Phagocytosis in the GI tract usually occurs in the FAE, particularly in M cells [33].

Paracellular pathways

Enterocytes of the epithelium are connected together by junctional complexes that include tight junctions (TJ), adherent junctions, desmosomes and gap junctions. TJ are presented as focal contacts on the plasma membrane [20]. These contacts correspond to continuous fibrils where fibrils on one cell interconnect with the fibrils on the adjacent cell [14]. These fibrils consist of numerous transmembrane proteins that include occludin [34], claudins [35] and members of the junctional adhesion molecule (JAM) protein family [36].

TJs serve as a gate, while transporting molecules towards the lumen or away from it, they also maintain cell polarity by preventing the diffusion of proteins and lipids from the apical to the basolateral side in the outer cell membrane [37].

As you can see the barrier function is a very complex and a delicate mechanism that plays an extremely important role in maintaining a balance in the GI tract. It involves multiple systems and cell types. The epithelial barrier leakiness and increase in intestinal permeability are directly associated with the TJ complex malfunction [38, 39]. Claudin-2 has been shown to be upregulated in CD, whereas TJ proteins ZO-1, 2 and 3, JAM-A as well as occludin and claudin-1 [40, 41, 42] are known to be downregulated in CD patients. TNF disrupts the TJ proteins thus inducing permeability via NF- κ B and MLCK dependent pathway in an apoptosis-independent manner. AIEC has been shown to disrupt TJ composition specifically targeting ZO-1, leading to an increased appearance of gaps between epithelial cells [43, 44]. However, some probiotics and commensal bacteria induce the upregulation of ZO-1, thus restoring the barrier [45].

A disturbed barrier function has been described in both animal and human models, it most often includes the malfunction of transcellular as well as paracellular pathways [14]. A disturbed barrier function can lead to the development of such diseases as an intestinal hypersensitivity [46], an irritable bowel syndrome [47, 48] and IBD [9] which includes among others Ulcerative colitis (UC) [49, 50] and CD [51, 52].

Crohn's disease

CD is a chronic relapsing inflammatory disease that can affect any part of the GI tract [51]. It is frequently characterized by abdominal pain, fever, diarrhea often with a mix of blood or mucus [53]. CD is a disease that affects the whole population worldwide.

The number of cases of CD varies geographically; however, there seems to be a higher number of incidents in the northern regions. A study conducted in 1996 concluded that the number of inflammatory bowel disease [9] incidents in Europe was 80% higher in the northern part compared to the central and southern parts of Europe [54]. Furthermore, a study of 380 CD patients in northern and southern centers has demonstrated that patients from the north have more severe disease conditions than those in the south [55].

The CD rates have always been higher in Western countries [56], an increase in CD incidents was observed in the 1970s, the same trend was observed in the developing countries, however, it was less pronounced there [57, 58]. A number of studies have been conducted in the USA [59], South Korea [60], Scandinavian countries [51, 61] and based on the data obtained one can conclude that

CD is slightly more predominant among women in their 30s who are smokers [62]. Within the first five years, at least, one third of the patients have to undertake surgery [63].

Etiology

Genetic factors

It has been confirmed that a genetic predisposition is one of the important factors that triggers the CD development. Over 100 CD associated genes/loci have been identified [64]. A number of polymorphisms that predispose to CD are highly expressed in the gut epithelia. Gene NOD2/CARD15 has been identified as the primary one associated with the development of this disorder. It belongs to the NOD like receptor family [65]. Furthermore, it has been shown that the balance in the regulation of NLRs and TLRs plays an important role in maintaining the intestinal homeostasis and defense against external pathogens [9]. A number of genes: NOD 2, ATG16L1 and IRGM altogether are involved in the bacterial sensing and clearance function, thus once a mutation takes place in these genes it might lead to defects in the anti microbial peptides (defensins) secretion [66, 67]. Other genes also known for their important role in the CD development are: XBP1 gene which is associated with the defects in Paneth cells function [68] and IBD5, MDR1 and DLG5 that are important in maintaining epithelial integrity, immune response and barrier function [69].

Barrier dysfunction

As it has been previously stated, the barrier function maintains a fragile balance between the nutrients absorption and the mucosa protection against pathogenic bacteria and viruses' penetration. In CD patients a barrier dysfunction is a combination of both the transcellular and the paracellular route [52, 70]. The dysfunction of the transcellular route manifests through a heightened protein antigens uptake that appears to happen due to the TNF secretion increase [71, 72, 73]. Furthermore, the paracellular permeability route is also impaired in patients with CD, under normal circumstances; it is an effective barrier against antigenic macromolecules, however, in CD patients an increased permeability of medium sized probes is observed in the small intestine [71, 74]. Moreover, structural changes and leakage of the tight junctions in response to luminal stimuli are observed in these patients [70, 75]. A reduction in the expression of a number of tight junctional proteins has also been observed in CD compared to healthy individuals. A decreased

expression of occluding, claudin 5 and claudin 8, and a less obvious increase in claudin 2 seem to be directly linked to a TNF high production [70, 76].

As it was previously mentioned, FAE is one of the important players in the barrier function. Interestingly, primary lesions in CD patients can most frequently be found in PPs [77]. The aphthous ulcer that is the result of M cells necrosis that overlie the PPs lymphoid follicles is considered to be the earliest CD lesions [78]. The peak age of the CD diagnosed people is the time when the highest number of PPs is formed in the intestine [79].

Environmental factors

Industrialization has greatly affected people's lives, their life style and diet. People concentrate more on their careers and work [47], the breastfeeding period is getting shorter [80], there appeared improved domestic conditions with better sanitation and less crowded environment [81], people are exposed to pollution and take to western diet of high amounts of sugar and polysaturated fats [82] as well as to increased tobacco use [83, 84, 85].

All of these factors play an important role in the development of the disease, however, tobacco use has been shown to significantly increase the risk of developing this disorder, surprisingly neither nicotine nor carbon monoxide seems to be the cause [86].

An increasing number of CD incidents in China, South Korea and Puerto Rico where traditionally the number of cases has been low is registered and it is linked to the introduction of western diet in these countries [87].

Microbiota

Microbiota is another factor that plays an important role in the development of CD. The intestinal microbiota composition can be affected by a number of factors such as antimicrobial drugs, vaccination and diet preferences [88]. The dysbiosis of intestinal bacteria is most often manifested as a change of an intestinal flora type, quantity, proportion, localization and biological characteristics, thus challenging the intestinal immune response. Dysbiosis induces the overgrowth of pathogenic bacteria in the gut and damages the intestinal mucosal barrier which leads to the increased intestinal permeability and opens up the entry way for pathogenic bacteria and its products into the intestinal lamina propria [89].

Dysbiosis and a high number of intramucosal bacteria that include adhesive species [90] are often found in CD patients [91, 92]. *Mycobacterium avium*, *Mycobacteria paratuberculosis* and

Yersinia pseudotuberculosis have been isolated from the tissue of both adults and children suffering from CD [93]. It has been shown that bacteria do not inhabit the mucosa of healthy intestine; however pathogenic bacteria have been found in the mucosa of CD patients, particularly *Escherichia coli* (*E.coli*) [94, 95].

Additionally, not only an increase in pathogenic microbiota can be observed in CD patients but also a decrease in the beneficial bacteria such as *Faecalibacterium prausnitzii* (FP), that is a member of the *Phylum Firmicutes* that possesses anti-inflammatory properties [96]. It remains unclear whether the dysbacteriosis is a primary cause of the disease or if it develops in the course of the disease. However, there is evidence that demonstrates that the intestinal microbiota can be shaped by the hosts genotype [97, 98] as well as by other factors including diet, habits, exposure to different infections and the use of antibiotics [99, 100]. It is important to point out that dysbiosis alone is not enough to cause CD.

CD is known to frequently occur after the patient has suffered from infectious gastroenteritis, however, the direct link between the two remains to be proven [101].

Figure 1. Etiology of CD.

The image is a schematic summary of the factors that contribute to the CD etiology.

Adherent invasive *E.coli* (AIEC)

E.coli is one of the numerous bacteria inhabiting the intestinal flora [102]. *E.coli* is a predominant aerobic gram-negative bacteria species of the normal intestinal flora; however, it can also be involved in a number of intestinal disorders. Diarrheagenic *E.coli* differs from those that normally inhabit the colon. They possess distinct virulence properties, such as the production of enterotoxins as well as cytotoxins. Moreover, it is prone to tissue invasion and has the ability to adhere to enterocytes. Different strains of *E.coli* possess a different virulence level depending on their virulence genes [103].

Numerous strains of *E.coli* bacteria have been tested to study whether they possess adherence genes, samples for this study were taken from healthy controls and UC and CD patients. The data suggested that there was an increase in the frequency of adherent *E.coli* isolates in UC and with CD patients but not in the control patients [104, 105, 106].

Studies have shown that there is an increased number of *E.coli* bacteria found in the feces of CD patients during the active phase of the disease [106, 107], *E.coli* antigen has been identified by immunohistochemistry in tissue from CD patients [107]. Invasive properties of certain bacterial species play a crucial role in their ability to colonize intestinal mucosa and induce inflammation [103]. One of the invasive types of *E.coli* that has been highly associated with CD is the adherent invasive *E.coli*. AIEC strains have been identified in 21% of the ileal samples obtained from the ileum of CD patients, compared to 6% of the ileal control group and in less than 5% of the colonic samples from both IBD and control patients [92]. AIEC strains are not exclusive for CD since it has been identified in the non-IBD control patients' mucosa as well. However, it is obvious that these bacteria preferentially colonize the CD patients' mucosa and are one of the factors that trigger inflammation in these patients [92].

A strain of *E.coli* bacteria named LF82 has been isolated from the ileal mucosa of CD patients. It has been detected in CD patients' populations in France [92], United Kingdom [94], Spain [108] and USA [109].

This strain is characterized as an adherent invasive strain. It efficiently invades intestinal epithelial cells *in vitro*. It has been revealed that its uptake is dependent on actin microfilaments microtubules and it has the ability to survive and replicate intracellularly in the host cell cytoplasm after lysing the endocytic vacuole. The invasive *E.coli* strain has been shown to survive and replicate inside

macrophages without causing the host cell's death in *in vitro* experiments [110]. The AIEC exact role has not yet been identified, however, it is safe to say that the AIEC strains have the ability to adhere and invade intestinal epithelial cells thus moving into deeper tissue and initiating the uptake by macrophages. Since AIEC have the ability to survive and replicate within the infected macrophages they cause an ongoing inflammatory process which in turn leads to a continuous recruitment of macrophages that potentially induce the formation of granulomas [107, 111]. It is worth noting that FAE plays a role of a portal for pathogens to penetrate the barrier and be introduced to the immune cells. Studies have shown that AIEC LF82 has the ability to interact with the murine derived PPs by using type 1 pili LPF [112]. It has been demonstrated that the number of AIEC increases during the course of the disease [113, 114]. Experimental studies have also found an increased uptake of non-pathogenic strains of *E.coli*: K12 and HB101 across FAE of CD patients compared to the non-IBD control patients and even compared to UC patients [115]. Other pathogenic bacteria, for example, *Salmonella Typhimurium*, *Shigella boydii* and *S. flexneri* are also known to use PPs as an entrance route to penetrate the intestinal barrier [116, 117].

E.coli strains have not only been isolated from the ileal mucosa of CD patients but have also been identified deep inside the CD patients colonic mucosa, one of colonic associated strains of *E.coli* is *E.coli* HM427 [118]. This strain has been isolated from the colon of CD patients and has been shown to have the ability to adhere and invade enterocytes as well as to penetrate deep into the mucus layer of the colon of CD patients [94].

Defensins

As it was previously mentioned, one of the GI tract defense mechanisms against the bacterial invasion is the secretion of antimicrobial peptides such as defensins. Defensins are one of the oldest elements of the immune system [119]. They are secreted by Paneth cells that are located at the bottom of the crypt [120]. Paneth cells secrete human α -defensin 5 and human α -defensin 6. Human α -defensin 5 is mainly expressed in the intracellular granules of Paneth cells. These antimicrobial peptides are secreted as a response to bacteria in the ileal lumen [120, 121, 122]. One of the most important functions of defensins is to shape the intestinal microbiota by removing pathogenic bacteria [123].

Human α -defensin 5 is a potent bacterial killer. Earlier they were thought to do so by destroying the bacterial membrane [119], however, this does not stand to be true. Recent studies have

indicated that human α -defensin 5 and human α -defensin 6 do not permeabilize bacterial membranes, instead they eradicate bacteria by localizing to the cytoplasm and possibly interacting with DNA [124].

Human α -defensin 5 reduced production is a central piece of the ileal CD in humans, as the pathogenesis of this disease may be caused by an altered mucosal antimicrobial defense or variations in the microbiota composition [125].

The mutation in the pattern recognition receptor nucleotide-binding oligomerization domain containing 2 (NOD2) has been associated with the reduction of human α -defensin 5 and human α -defensin 6 in the ileal CD in humans [66].

Other studies have also revealed that a mutation in the granule exocytosis pathway factor ATG16L1 [126, 127] or in the endoplasmic reticulum stress response gene XBP1 [128] [68] leads to an increase in the Paneth cells apoptosis which is associated with CD. Moreover, increased autophagy of Paneth cells and a reduced number of secretory granules in CD patients have also been observed [129].

Figure 2.

A schematic image of the intestinal barrier.

The image represents FAE and VE located in the distal ileum as well as the layers, main cells and structures involved in the intestinal barrier function in the ileum.

TNF

There exists a complex interaction between epithelial cells, immune cells and foreign bodies that transition along the gut in the GI tract. A constant communication process between epithelial and immune cells maintains the intestinal barrier. The gut-associated lymphoid tissue generates an immune response against pathogens or a clinical immune response towards dietary and microbial antigens [130].

One of the important factors of the CD etiology is a malfunction of the barrier function. An impaired barrier function leads to an increased exposure of the mucosal innate and acquired immune system to pro-inflammatory molecules, thus causing continuous constant inflammation [24]. Among immune dysregulation, it is important to point out an abnormal involvement of activated T cells and macrophages. Once these immune cells are activated they produce high amounts of pro-inflammatory cytokines including IL-1, IL-6 and TNF as well as reactive nitrogen and oxygen metabolites, at the same time the production of anti-inflammatory cytokines is decreased. This leads to recruiting of neutrophils and monocytes into the gut mucosa promoting inflammation. And it, in turn, leads to tissue damage, increased permeability and leakiness [131, 132, 133, 134]. During active CD macromolecules can penetrate the barrier at a high rate via the breaks in the integrity of the epithelium that are caused by the inflammatory process as well as through the M cells in the terminal ileum that are increased in number due to the inflammatory process [135]. One of the main pro-inflammatory cytokines to induce inflammation is TNF. It has been demonstrated that CD patients have elevated levels of TNF in the mucosa [136], faeces [137] and in the serum [138].

TNF is known to increase paracellular permeability via the effects on TJ proteins [139]. There is evidence demonstrating that TNF can also induce the bacterial passage across intestinal epithelial cells *in vitro* [140]. However, it is not clear whether CD causes an increased permeability or whether it precedes the disease.

One of the traditional treatments of CD patients is administering a monoclonal anti-TNF antibody.

Infliximab is a monoclonal IgG1 antibody that can bind and neutralize soluble and membrane bound TNF. Although the infliximab complete mechanism is not yet fully understood studies have revealed that CD patients treated with this drug showed improvement in permeability to small

molecules [141], a normalized rate of epithelial cells apoptosis [70], as well as induced apoptosis in T lymphocytes and macrophages, thus demonstrating a strong anti-inflammatory effect [142].

CEACAM6

CEACAM family members are cell membrane associated glycoproteins that are part of the immunoglobulin superfamily. CEACAM1, CEACAM5 and CEACAM6 are expressed on intestinal epithelial cells, CEACAM6 attaches to the epithelial cell membrane via a GPI anchor [143]. CEACAM5 and CEACAM6 have been seen to be upregulated in epithelial derived tumors from colorectal cancer patients [144]. The CEACAM6 upregulation has been observed in the ileum of CD patients. Moreover, studies have shown that CEACAM6 serves as a receptor for AIEC via type 1 pili [94, 145]. No CEACAM6 expression upregulation was observed in the CD patient colonic mucosa, and there was no increase in the CEACAM6 expression in the UC patients' colon [145]. These findings are a plausible explanation of the increased number of AIEC associated with the CD ileal mucosa [146]. Furthermore, studies have shown that transgenic mice strain CEABAC10 that express human CEACAMs when infected with LF82 bacteria demonstrated a threefold increase in an intestinal transcellular permeability and disrupted a mucosal integrity in type 1 pili-dependent mechanism. However, no impact on permeability was observed when mice were infected with non-pathogenic *E.coli* [147]. A study has shown that when primary ileal enterocytes isolated from CD patients are pre-treated with anti-CEACAM6 antibody the ability of LF82 bacteria to adhere to these cells is strongly reduced [145].

The AIM

The aim of the present thesis was to study bacterial epithelial interaction during inflammation in *in vivo*, *ex vivo* and *in vitro* models.

Specific Aims

- To investigate into the effects of *Faecalibacterium prausnitzii* supernatant treatment of intestinal barrier in an *in vivo* model of DSS induced colitis in mice.
- To determine how infliximab affects uptake of adherent *E.coli* HM427 across the colonic mucosa in CD patients and in an *in vitro* model.
- To examine the mechanisms of transepithelial transport of *E.coli* LF82 in the CD mucosa and in an *in vitro* FAE model
- To study the involvement of human α -defensin 5 in the defense mechanisms against LF82 in CD mucosa and in an *in vitro* model of FAE.

Materials and Methods

Paper I

Mice

All the animals that have been used in this study were acclimatized for a minimum of one week prior to performing the experiments. Animals were kept under constant housing conditions (12 hour light/dark cycle 55±5% humidity and 22±1°C); the mice had free access to water and food throughout the whole period of the experimental procedure. The mice were housed three per cage in individually ventilated caged 7dm³ (37cmx16cmx13cm). All the cages were cleaned and the bedding was changed weekly. The study was approved by the ethical committee on animal experiments at Linköping University, Sweden.

A total of 56 female C57BL/6 mice (Scanbur BK AB, Sollentuna, Sweden) were used. The choice of this strain was from the previously published papers on Dextran sodium sulfate (DSS)-induced colitis [148].

The study was approved by the ethical committee on animal experiments at Linköping University, Sweden.

In vitro permeability studies

In order to study the uptake of bacteria across the epithelial cell monolayer a cell line of human colon adenocarcinoma cell line Caco-2 cells (ATCC, ML, USA) and Caco-2-clone 1 (c11) (received as a kind gift from Maria Rescigno, Milan, Italy) were used. When cultured under specific conditions Caco-2 cells differentiate into enterocyte that express tight junction proteins and microvilli thus morphologically and functionally resembling enterocytes found in the small intestine.

In order to study bacterial transport of AIEC across FAE in *in vitro* model Caco-2-c11 cells were used. This subclone is best suited to be differentiated into M like cells when co-cultured with Raji B cells. Monoculture of Caco-2-c11 cells was used as an *in vitro* VE model – control group.

Paper II

Caco-2 cells were cultured on 3µm polycarbonate filters. Upon reaching confluence cells were pre-treated with TNF for 24h prior to infection with HM427 bacteria, to mimic the immune response that takes place in the body in *in vitro* conditions. To further research into the mechanisms behind the bacteria cell interaction under the influence of a widely used anti-TNF monoclonal

antibody – infliximab cells were exposed to endocytosis inhibitors. The endocytosis inhibitors – methyl- β -cyclodextrin (m β cd) and cochicine were added on the apical and basolateral side giving us the opportunity to study the mechanism behind the bacteria cell interaction in *in vitro* conditions mimicking an *in vivo* situation (Figure 3).

Figure 3. Schematic image of the in vitro experimental set up

Illustration of the in vitro set up. A schematic image of the cells treatment that has been performed in the present study. Presenting Caco-2 cells that have been exposed to TNF on the basolateral side, endocytosis inhibitors on both the apical and the basolateral side followed by an HM427 infection on the apical side.

Paper III and IV

An *in vitro* FAE model has been set up to study bacteria cell interaction. In order to do so a modification of the co-culture model of FAE, which was originally described by Kerneis *et al.* was used. To achieve this model Caco-2-cl1 cells were co-cultured with Raji B cells (ATCC, ML, USA). The transformation of Caco-2-cl1 cells into M like cells was confirmed by two methods. Firstly, the transformation was established by measuring the transepithelial resistance (TER). The transformation was confirmed if the TER in the co-culture model was at least 10% lower compared to the monoculture model. Secondly, once the transformation was confirmed by the TER measurement cells would be infected by *Salmonella Typhimurium*. If the passage of the bacteria were higher in the co-culture model compared to the monoculture model the transformation would be confirmed (Figure 4).

Figure 4. Schematic illustration of the in vitro FAE model set up

Illustration of the in vitro FAE model and experimental set up. Specifically the process of the co-culture. The first step was culturing Caco-2-cl1 cells until they reach confluence, further Raji B cells were added on the basolateral side. Cells were co-cultured until the transformation of Caco-2-cl1 cells into the M like cells took place. The last step was the infection of cells on the apical side by LF82.

Bacteria

The present study was aimed at investigating the uptake of pathogenic bacteria *Salmonella Typhimurium* and AIEC strains that have been previously isolated from the mucosa of CD patients HM427, LF82 and the mutant strain lacking the *lpfA* operon - LF82^{ΔlpfA}.

HM427 bacteria were transformed with plasmid pEGFP (BD Biosciences), for expression of the enhanced green fluorescent protein (EGFP). LF82 bacteria were transformed using pPFV25.1 to make the bacteria express green fluorescent protein (GFP) [149]. The mutant strain of LF82 bacteria lacking *lpfA* gene was produced with a PCR product using the method that was previously described by Datsenko *et al.* [150].

The transfection of *Salmonella* was carried out by adding 5ml of plasmid enhanced green fluorescent protein 1:100 to 50 ml of competent *Salmonella*. The mixture was gently shaken on ice for 30min followed by heat shock at 40°C for 90s. After addition of 450 ml SOC medium, bacteria were incubated on shaker at 200 rpm at 37°C for 1h. The bacteria were then plated on LB ampicillin agar plate and were grown at 37°C.

In paper II

Tissue samples were obtained from 7 CD patients with colonic location at the median age of 30 years old (range 24-37). The included patients had a clinical indication for treatment with the anti-TNF therapy. Colonic biopsies were acquired from the macroscopically non-inflamed sigmoid colon during ileocolonoscopy prior to initiating the treatment with Infliximab (anti-TNF

treatment). Tissue samples were obtained from the same patients after 2 weeks of treatment under the same procedure.

Biopsies from 8 healthy individuals were taken (control group). This group included 4 healthy volunteers and 4 otherwise healthy individuals that were undergoing control colonoscopy for colonic adenomas. The median age of the healthy individuals was 36 years (range 25-81). Biopsies were taken from the sigmoid colon.

The studies were approved by The Regional Ethical Review Board, Linköping, Sweden; all subjects gave their written informed consent.

In paper III

The tissue samples were obtained from 25 patients with CD and from 25 patients with colon cancer (non-IBD control group). The specimens were obtained from the neo-terminal ileum, or terminal ileum next to the ileocaecal valve during surgery on CD patients. The median age of the patients was 49 years old (range 16-81). All patients were in an active phase of the disease according to the Montreal classification.

The non-IBD patients (control group), median age of 74 (range 46-88), patients had no generalized disease and none had received pre-operative chemo- or radiotherapy.

The studies were approved by The Regional Ethical Review Board, Linköping, Sweden; all subjects gave their written informed consent.

In paper IV

Tissue samples were obtained from 11 patients with CD and 11 patients from non-IBD colon cancer patients used as the control group. The specimens were derived from the terminal ileum next to the ileocaecal valve or from the neo-terminal ileum from patients with a previous resection. The median age of the CD patients was 57 years old (range 22-70). All patients were in an active phase of the disease according to the Montreal classification.

The non-IBD colon cancer patients used as a control group were of median age of 74 years old (range 53-83). The patients had no generalized disease and none had received pre-operative chemo or radiotherapy.

The studies were approved by The Regional Ethical Review Board, Linköping, Sweden; all subjects gave their written informed consent

Permeability markers

⁵¹Cr-EDTA probe was used as a paracellular marker in the Ussing chamber experiments. The EDTA molecules are known to pass between the cells via the paracellular route. In order to track the passage of EDTA molecules they are bound to radioactively labelled Cr. The binding is very strong, thus assuring that the passage of Cr will be equal to the passage of EDTA. ⁵¹Cr-EDTA was added on the mucosal side, an aliquot was collected from each sample, and the passage of the radioactive probes was measured in the gamma counter.

Ussing chambers

The Ussing chamber technique was used to study permeability across tissue samples. The tissue was obtained during surgery and the biopsies from CD patients and non-IBD colon cancer patients. VE and FAE were identified using a dissection microscope. The samples were mounted in the modified Ussing chambers (Harvard apparatus Inc., Holliston, MA, USA). The mucosal compartment was filled with 1.5 ml cold 10mM mannitol in Krebs buffer and the serosal compartment was filled with 10mM glucose in Krebs buffer. The chambers were maintained under constant temperature of 37°C and were continuously oxygenated by 95% O₂/5% CO₂ and circulated by gas flow. The Ussing chambers were equilibrated for 40 min in order to achieve a steady state conditions. Barrier properties were studied after the equilibration period was complete. The condition of the tissue was controlled in open circuit conditions by monitoring the transepithelial potential difference (PD), short-circuit current (I_{sc}) and TER which was registered every second minute with the help of Ag/AgCl electrodes with agar salt bridges and one pair of current giving platinum electrodes. Bacteria and ⁵¹Cr-EDTA were added on the mucosal side to study transcellular and paracellular permeability (Figure 5).

Figure 5. Schematic illustration of the Ussing chamber

The images include a photo and a schematic presentation of the Ussing chamber that was used in the presented projects.

Confocal and Fluorescent Microscopy

In paper II

Confocal microscopy was used to study the HM427 bacteria-lipid raft interaction in Caco-2 cells.

The cells were cultured on transwell filters, stimulated with TNF and infliximab on the basolateral side, pre-treated with m β cd and further infected with *E.coli* HM427. The cells were further fixed in PFA, stained for F-actin and lipid rafts formation. Confocal microscopy images were taken with Zeiss LSM 710 laser confocal microscope (Jena, Germany), using a 60x oil objective. Z-stack images were acquired, and used to study co-localization of lipid rafts with HM427 bacteria. This was done using Huygens Professional software (Scientific volume imaging BV, The Netherlands).

In paper III and IV

The cryosections from CD patients and non-IBD control patients' tissue were stained to study the expression of the CEACAM6 receptor (Paper IV) and the expression of human α -defensin 5 (paper III). Images of the sections were acquired using a Nikon Eclipse E800 (Amsterdam Netherlands) fluorescent microscope equipped with the Nikon DS-Ri 1 (Amsterdam Netherlands) digital camera. The expression of CEACAM6 and human α -defensin 5 were evaluated using Image J software (National Institute of Health).

Results

Paper I

Paper 1 focuses on the effects of anti-inflammatory bacteria on the intestinal colonic inflammation in a mouse model. To perform this study a commensal bacterium *Faecalibacterium prausnitzii* (FP) which has been previously shown to be underrepresented in the microflora of Crohn's disease patients has been used. The mice were divided into two groups. One group received 3% DSS in their drinking water for five days to induce acute colitis. From day three of the experiment and the following seven days the mice received daily gavage with supernatant from FP while only culture broth was given to the controls. At the end of the experimental procedure the ileum and colon tissue was harvested and permeability studies using the Ussing chamber technique were performed. The data obtained showed a significant increase in ^{51}Cr -EDTA passage across the ileum of the mice with DSS colitis. The passage was significantly lower in the group of mice that received FP supernatant (Figure 6). No significant effect could be observed in the colon tissue.

Figure 6. Passage of ^{51}Cr -EDTA across intestinal tissue from mice with DSS colitis

The passage of ^{51}Cr -EDTA across ileal FAE (A), VE (B) and colon (C) epithelium obtained from mice. The data in figure 1 A, B and C is presented as mean $\pm$ SEM. The data was analyzed using Anova and Bonferoni's tests to study statistical significance. The data in figure 1 C is presented as median $\pm$ IQR, Kruskal-Wallis test was performed to study statistical significance (** $p \leq 0.01$, # $p \leq 0.05$). The number of mice was 8-12 per group.

The tissue from mice was also saved in order to perform western blot analyses to study the expression of the TJ proteins: claudin-1 and -2. The data showed a significantly higher expression of claudin-1 in the DSS treated group compared to the control group. Furthermore, a numerically higher expression of claudin-2 was observed in the DSS group compared to the control group (Figure 7).

Figure 7. Densiometric analyses of Claudin 1 and 2 expression performed by western blot.

The collected tissue was used to study the expression of claudin 1 and 2 by western blot analyses.

The densiometric measurements were performed using Image J software.

The data has been normalized against the β actin loading control. The data is presented as median $\pm$ IQR. Statistical significance was tested using Mann-Whitney test. $n = 4$ mice per group.

Conclusion

The obtained data suggests that FP improved the intestinal barrier function by affected paracellular permeability in the present mice model. Therefore, we can conclude that in the present model FP reduces the inflammatory process in the DSS induced colitis mouse model.

Paper II

This study is aimed at determining the effects and mechanisms of infliximab on the uptake of colonic CD associated *E.coli* HM427 across the colonic mucosa in *in vitro* and *ex vivo* models.

The first step of the study was to identify the effects of infliximab on bacterial transcytosis across colonic biopsies. The biopsies were obtained from CD patients before and after treatment with infliximab. Healthy volunteers and patients who came in for polyp scanning were used as a control group. The transcytosis of HM427 was studied using the Ussing chamber technique. The data showed a significant decrease in the uptake of HM27 across the colonic biopsies of CD patients after they received an infliximab treatment.

The next step was to study the mechanisms behind the infliximab positive effects on the barrier function that has been shown in the *ex vivo* experiments. To do so an *in vitro* model was set up. Caco-2 cells were cultured on polycarbonate filters and were pre-treated with TNF on the basolateral side for 24h. Further, the cells were treated with an inhibitor of lipid rafts formation – mβcd. The cells were further infected with the HM427 bacteria. The data has shown that the passage of the bacteria was reduced by 50% while the internalization was reduced by 80% in the cells exposed to the mβcd compared to the cells that had not been exposed to the inhibitor. However, no effect of colchicine (microtubule formation inhibitor) has been observed (Table 1).

Table 1 Transepithelial uptake and epithelial cell internalization of AIEC HM427 in TNF-exposed Caco-2 monolayers

	TNF (Fluorescence Intensity)	TNF + infliximab % of TNF	TNF + mβcd % of TNF	TNF + colchicine % of TNF
Bacterial uptake	2768 (2009- 4015) (100%) n=9	78.9 (69.25- 95.54)** n=9	49.7(25,77- 60.77)** n=5	116.7(90.32- 129.8) ^{ns} n=5
Internalization of bacteria	651.9 (415- 1076) (100%) n=10	90.77 (84,73- 96,24)** n=9	82.79(60,47- 92,32)** n=5	95.54(78.5-106.9) ^{ns} n=5

Values are presented as median (25-75th percentile). * p<0.05, **p-value<0.01, ns – not significant versus TNF. n- number of experiments performed in triplicates on separate occasions

Conclusion

The data obtained suggests that infliximab restores the barrier function and decreases the transcytosis of HM427 across the CD colonic biopsies. Furthermore, infliximab affects the uptake and internalization of AIEC HM427 in the colonic mucosa in *in vitro* and *ex vivo* models and the effect is partially mediated by blocking of the lipid rafts formation in epithelial cells

Paper III

This study deals with the uptake of AIEC ileal derived *E.coli* LF82 through the ileal mucosa of CD patients, specifically FAE. In addition, the role of the CEACAM6 receptor in this process is investigated.

An *in vitro* FAE co-culture model was set up to explore the transport of LF82 bacteria. The cells were infected with LF82 bacteria and fluorimetry was used to study the passage of these bacteria. The data demonstrated a significantly increased LF82 bacteria passage across the *in vitro* FAE model compared to *in vitro* VE (Figure 7). The cells were further pre-treated with the anti CEACAM6 antibody prior to infection, thus blocking the CEACAM6 receptor. The results showed a significant decrease in the bacterial passage across the cell monolayers after blocking the CEACAM6 receptor.

Figure 8. Passage of *E.coli* LF82 bacteria across *in vitro* FAE

Passage of *E.coli* LF82 in Caco-2-cl1 cells and the co-culture model measured by fluorimetry.

A) Cells were exposed to LF82 bacteria alone. **B)** Cells were pre-incubated with a monoclonal anti-CEACAM6 antibody prior to infection. The data is presented as median (25th-75th interquartile range) and statistical significance was estimated using Mann-Whitney U test, * $p < 0.05$, ** $p < 0.005$.

The LF82 bacteria passage was also studied in an *ex vivo* set up on the human tissue samples obtained after surgery. The ileal FAE and VE samples from CD patients and non-IBD control were used in the Ussing chambers to study the transport of LF82 (Fig. 8 A). The data revealed that the AIEC LF82 passage was significantly higher in the FAE compared to VE, and this passage was significantly reduced once the tissue was pre-treated with the anti-CEACAM6 antibody (Figure 9 B).

Figure 9. Effects of a monoclonal anti-CEACAM6 antibody on live green fluorescent protein-labeled *E.coli* LF82 in the follicle-associated (FAE) and Villus epithelium (VE) of patients with Crohn's disease (CD) and non-IBD controls.

Tissue samples of FAE were mounted in the Ussing chambers. The LF82 bacteria were added on the mucosal side, with or without 20 min pre-treatment with the human anti-CEACAM6 antibody. The LF82 bacteria passage was measured by fluorimetry and re-calculated as bacteria/chamber x 10³. The data is presented as median (25th-75th interquartile range) and statistical significance was estimated using Mann-Whitney U test, *p < 0.05, **p < 0.005.

Conclusion

Given the data obtained in the course of this study we can conclude that LF82 favors FAE as an entry path to penetrate the intestinal barrier and the CEACAM6 receptor plays a crucial role in its ability to do so.

Paper IV

This study looks into the effect of human α -defensin 5 on the transport of AIEC LF82 across FAE of CD patients and non IBD controls in an *in vitro* model and *ex vivo*.

An *in vitro* co-culture model was set up to study the impact of human- α defensin 5 on the LF82 bacteria passage across FAE. The acquired data showed that the LF82 passage in the *in vitro* FAE model was 28.9% higher than in the *in vitro* VE model (Figure 10). Moreover, the data showed that the pre-treatment of cells with human α -defensin 5 caused a slight decrease of 3.6% in the *in vitro* VE model. However, the human α -defensin 5 treatment reduced the passage of bacteria across *in vitro* FAE model by 28.7% compared to the untreated cells (Figure 10).

In addition, a thorough study was made of the LF82 bacteria passage across the FAE tissue obtained from CD patients and non-IBD control patients undergoing surgical procedures. The passage of bacteria was investigated in an *ex vivo* set up in the Ussing chamber experiments. The data revealed a significant increase in the uptake of the LF82 bacteria across the FAE of CD patients and non-IBD control compared to the LF82 passage across these patients VE. However, the tissue pre-treatment with human α -defensin 5 significantly decreased the LF82 passage compared to the passage through the untreated tissue in both CD and non-IBD control patients (Figure 11).

Figure 10. Passage of *E.coli* LF82 bacteria across *in vitro* FAE

Passage of *E.coli* LF82 in Caco-2-cl1 cells and the co-culture model measured by fluorimetry. The data is presented as median (25th-75th interquartile range) and statistical significance was estimated using Mann-Whitney U test, * $p < 0.05$, ** $p < 0.005$.

Figure 11. Effects of human α -defensin 5 on live green fluorescent protein-labeled *E.coli* LF82 in the follicle-associated epithelium (FAE) of patients with Crohn's disease (CD) and non-IBD controls.

The FAE tissue samples were mounted in Ussing chambers, LF82 bacteria were added on the mucosal side, with or without 20 min pre-treatment with human α -defensin 5. The passage of LF82 bacteria was measured by fluorimetry and re-calculated as bacteria/chamber $\times 10^3$. The data is presented as median (25th-75th interquartile range) and statistical significance was estimated using Mann-Whitney U test, * $p < 0.05$, ** $p < 0.005$.

Conclusion

The obtained data has confirmed the findings discovered in the previous study that the LF82 bacteria favor FAE as a way to penetrate the intestinal barrier. Furthermore, it has shown that human- α defensin 5 plays a crucial role in reducing the AIEC LF82 uptake.

Methodological considerations

Cell culture

Cell culture is a very widely used technique in many fields. The beauty of using cell lines is that you can perform the experiments in a simple and control environment. More than that you avoid being dependent on availability of human tissue, since as long as you have a cell line growing, it is easy to set up an experiment at any convenient time. There are many cell lines available to suit a large variety of studies. Co-culturing cell lines as has been done in Paper III and IV gives us further opportunities to broaden our research by creating new *in vitro* models.

An *in vitro* co-culture model that was used in Paper III and IV gave us the opportunity to study the passage of bacteria in *in vitro* conditions across FAE at any given time (since we were not bound to the time of the next surgery). However, while working with cell lines one should be aware of some obvious drawbacks which we can't help mentioning.

First, when working with cells you only use one component of the whole system, thus losing the complex interactions of different components that are found in the body. Unless you use a 3 D cell culture model the plane of the study is very one-dimensional.

Second, Caco-2-c11 cells do not spontaneously express the CEACAM6 receptor (which was an important part of the study). It happens only in response to the TNF treatment or in response to *E.coli* infection but not without these stimuli. This made us strongly consider the best way to set up our experiments since we wanted to block the CEACAM6 receptor.

Third, cells are also sensitive to the outside environment, thus one should be very careful when handling them.

Fourth, working with cell lines requires working in sterile conditions, meaning that the slightest mistake can lead to contamination.

Finally, you have to be considerate of the time you handle cells outside the incubator since that can affect their response.

Ussing chambers

Ussing chambers are a unique technique that gives us the ability to study: tissue viability, paracellular and transcellular permeability, using different molecules or microorganisms. The Ussing chamber technique gives us a possibility to study permeability of the tissue derived from humans or animals that no other technique is able to provide so far.

We are also able to monitor tissue parameters such as transepithelial resistance and potential difference, all in one experiment. Moreover, due to the way that the tissue is exposed to nutritious solutions, oxygen and a constant optimal temperature we have the ability to keep the tissue viable for an extended amount of time.

The Ussing chamber technique gives us the possibility to perform experiments on tissue from human intestine obtained from unique patients. Moreover, any part of the intestine can be studied once we can receive surgically acquired specimens or biopsies. When using surgically obtained tissue we have the possibility to identify FAE and VE thus significantly extending our research possibilities.

Obviously, like any technique the Ussing chamber technique also has its setbacks. For one, the tissue used in this method is stripped of the muscle layer and has no connection with the nervous or endocrine systems, so similarly to the cell culture method we are removing one player from a giant field. However, in case of the Ussing chamber experiments the limitation is not so narrow as with the cell culture technique, because in this case you get a wide variety of the cells forming the epithelium as well as the mucosal layer.

Another limitation of this technique or, I should say, of working with tissue itself is the tissue viability. Since the moment the tissue is removed from the body during surgery or colonoscopy there is a short window during which the tissue is viable and the experiments have to be carried out. Our group has shown outside of this thesis that the obtained tissue maintains ATP levels up to 1h after being removed from the body if preserved in an ice-cold oxygenated buffer [151, 152]. Under normal circumstances the tissue is delivered to the lab within 30 min after being extracted. The duration of the experiments does not exceed 4h including the calibration time. However, other research groups have shown that it is possible to perform experiments up to 20h if FBS is added to the chambers for extra nutrition [153].

Despite the drawbacks of the Ussing chamber technique it provides an opportunity to perform a wide range of experiments on human as well as animal tissue.

Patients

In paper II, III and IV we used CD patients as a group of interest and colon cancer patients as a non-IBD control group. Colon cancer patients were our best choice for this study since it is impossible to acquire tissue specimens from healthy people. Although they do not meet the ideal parameters that we would want since generally colon cancer patients are older than the CD patients used in our studies. Moreover, since we studied the involvement of the CEACAM6 receptor in the AIEC ability to penetrate the barrier it would have been better to use non-cancer patients. The reason for that is that some colon cancer patients have an elevated expression of CEACAM6 receptor due to their condition. The use of this patient group as a control group was the only choice possible in our research, since the studies had to be performed on FAE and it is impossible to obtain this type of tissue specimens from healthy volunteers.

Discussion

This thesis has combined projects in which we have studied two out of four factors that are known to influence the development of CD: Immune dysregulation (paper II and III) and microbiota (paper I and IV). The interface between these two factors lies in the mucosal barrier.

Paper I

The project focused on studying the effects of commensal anti-inflammatory bacteria on inflammation in an *in vivo* colitis model. Commensal bacteria are known to have beneficial effects on reducing inflammation in CD patients [154]. Our research focused on the effects of *Faecalibacterium prausnitzii* (FP). It has previously been shown that FP is underrepresented in CD [91, 155, 156, 157]. That is why it was of great interest to study the effects of these bacteria strain on inflammation in an *in vivo* colitis model. A DSS induced colitis in mice was used as a colitis model in this study. The data obtained in this study demonstrates that FP has a positive effect on the barrier function that has been disrupted by DSS in an animal model by reducing paracellular permeability. It is important to point out that even though the same trend of improved paracellular permeability was observed through both the colon and the ileum of the DSS treated mice, the effect was stronger in the ileum. These results were somewhat unexpected but we hypothesize that it occurs due to the fact that the inflammation levels were higher in the colon tissue compared to those of the ileum and it caused thickening of the colonic tissue which reflected on the results.

In the present study we demonstrate that it has a positive anti-inflammatory effect and is able to improve the barrier function and an anti-inflammatory effect, it has previously been shown that FP is underrepresented in CD patients [155]. These facts lead us to believe that FP is involved in the CD pathogenesis. Moreover, we believe that FP can be considered as part of the potential future treatment for decreasing inflammation in CD patients.

Paper II

This project dealt with a CD immune aspect, specifically with the suppression of the high expression of TNF levels in CD patients. It also examines the effects of the anti-TNF therapy both at a clinical level as a TNF blocker and at a mechanistic level by looking at how it effects the bacteria-cell interaction and barrier function.

TNF is an important participant in CD, it plays an important role in the inflammatory process that takes place during CD and also it is known to be disruptive to the barrier function [158, 159].

Infliximab a monoclonal anti-TNF antibody based drug has been used for twenty years as an anti-inflammatory treatment for CD patients [70, 141]. The reason for this drug's success is due to the fact TNF plays a very important role in maintaining the inflammation and barrier function distraction during CD [158, 160].

The acquired results showed that the infliximab treatment causes a significant reduction of the HM427 passage across colonic biopsies compared to the passage before the treatment. The observed levels were nearly as low as those in the control group. The same pattern was observed when studying paracellular permeability using the marker ^{51}Cr -EDTA. The results showed that the increased paracellular permeability in CD patients' biopsies was significantly reduced by the infliximab treatment. These findings suggest that TNF does not only play a major role in increased intestinal permeability in CD patients, but also has a very strong ability to disrupt the epithelial barrier to bacteria. These findings are in line with previous studies that have shown a correlation between the reduction in the tight junctional integrity and an increased secretion of proinflammatory cytokines which, in turn, is associated with an increased bacterial penetration through the barrier *in vitro* [140] and *in vivo* [161].

Using an *in vitro* model of Caco-2 cells we were able to study the effects of infliximab on the epithelial barrier integrity. The results showed that treatment of cells with TNF for 24h significantly reduced TER, which is traditionally associated with the Caco-2 cells increased permeability. Interestingly, the treatment with infliximab was able to restore the transepithelial resistance in these cells. These findings are also in line with some previous studies that have demonstrated that adalimumab (another anti-TNF antibody) restores the damage to tight junctional complexes caused by TNF exposure in the Caco-2 cell monolayer [141].

We were further interested to see how exactly infliximab affected the bacterial cell interaction process. The results of the experiments performed in an *in vitro* model have demonstrated that both internalization and transepithelial transport of HM427 were significantly reduced by the infliximab treatment of cells that have prior received TNF, thus making us believe that by reducing the TNF levels infliximab both reduces the inflammation and the ability of pathogenic

bacteria to invade epithelial cells. This data also suggests that TNF does not only exploit tight junctional complexes but also affects the transcellular pathway to increase the bacterial uptake by Caco-2 cells. Enterocytes rarely internalize bacteria under normal conditions, but there appears more and more evidence that the bacteria cell interaction is a complex process that involves many aspects. During these interactions, bacteria can manipulate cell pathways that are involved in the inflammatory process [7, 162] as well as cell growth. This way it can affect both paracellular permeability and endocytosis.

However, the question remained of how exactly infliximab affected the cell bacteria interaction. In order to determine this we have used colchicine (an endocytosis inhibitor that blocks the microtubule formation) and mβcd (a lipid raft formation inhibitor). The data showed that colchicine had no significant effect on the TNF-induced transport or internalization of the HM427 bacteria. However, mβcd significantly decreased both the internalization and the passage of the HM427 bacteria across Caco-2 cells. *In vitro* studies have revealed that bacteria may exploit the lipid rafts root to gain access into the cells [163]. In fact, *E.coli* was one of the first bacteria shown to invade cells via the lipid raft pathway [164]. The importance of lipid rafts in the bacterial-cell interaction in the present study is further supported with our findings revealing that TNF and infliximab also affected co-localization of AIEC HM427 and lipid rafts. The co-localization was increased in the cells pre-treated with TNF, however, the levels of co-localization in the cells that received both TNF and infliximab was reduced to the levels of the control group. These results are in line with the previous studies that have shown that lipid rafts play an important role in bacterial uptake and transport through epithelial cell monolayers [140]. However, the novelty of the present study is clearly demonstrating that infliximab has beneficial effects in the barrier function on the uptake of AIEC HM427 induced by TNF via the lipid rafts formation. But mention should be made that in the present study these results hold true only to colon-associated AIEC HM427 since experiments have only been performed using this strain. Nevertheless, we could hypothesize that these data will be confirmed for other adherent strains as well since the infliximab treatment is known to downregulate the adhesion molecules needed for adherence and invasion of bacteria in the intestinal epithelium [165].

Paper III and IV

Microbiota plays an important role in the development of CD. The LF82 bacterium is adherent invasive *E.coli* that has been isolated from the ileum of CD patients [92]. In these two projects we were interested to look at the passage of the LF82 bacteria through the FAE of CD patients and non-IBD controls since it has previously been shown that the LF82 bacteria have a higher adherence to the ileal enterocytes of CD patients compared to the control patients. It has also been found that *lpf* [112] operon plays an important role in the LF82 ability to adhere and penetrate ileal enterocytes. The present project examined the interaction of LF82 and FAE as well as the role of *lpf* in the ability of these bacteria to invade and pass the barrier of FAE of CD patients. Also we were interested in studying the involvement of the CEACAM6 receptor in the LF82 bacteria uptake.

The first step was to make a study of the bacterial-epithelial cell interaction *in vitro*. To do so an *in vitro* FAE model was set up which is a co-culture model of Caco-2 cl1 cells and Raji B cells. Using this model we were able to investigate the LF82 passage across *in vitro* FAE, as well as the passage of mutant bacteria LF82 lacking *lpf* operon (LF82 Δ *lpfA*). We also researched into the involvement of the CEACAM6 receptor in the LF82 uptake. The *in vitro* data showed a significantly higher passage of the LF82 bacteria in the co-culture model compared to the monoculture. Moreover, the passage of LF82 Δ *lpfA* was significantly reduced in the co-culture model compared to the conventional LF82. To study the involvement of the CEACAM6 receptor in the LF82 uptake the epithelial cell monolayers were treated with an anti-CEACAM6 antibody prior to infection, this way blocking the binding sites. The results demonstrated a significant decrease in the passage of LF82 bacteria in the *in vitro* FAE model in cells that received the anti-CAECAM6 antibody. This data suggests that not only do the LF82 bacteria use FAE as an access route to penetrate the intestinal barrier but they also do so via the CEACAM6 receptor and its adherence ability is increased by the *lpf* operon.

It was of great interest to take the study to the next level and to repeat these experiments in *ex vivo* conditions using human tissue in Ussing chambers. The data obtained showed a significantly increased passage of LF82 across the FAE and of CD patients compared to the control group as well as a significantly higher passage of LF82 across FAE compared to VE in both groups, confirming our *in vitro* findings that the LF82 bacteria use FAE as an access route to penetrate the

barrier. The fact that the uptake was significantly higher in the CD patients was expected because it has been proven that the barrier function of CD is impaired thus providing an easier access for pathogens to penetrate the barrier.

We further looked at the mechanisms by which LF82 is able to penetrate the ileal mucosa. The data showed a significant reduction of the passage of LF82^{ΔlpfA} in FAE of CD and non-IBD patients, however, no significant difference was observed in the VE. Moreover, we showed a significant decrease in the passage of the LF82 bacteria in the chambers that received anti-CEACAM6 as compared to the control chambers. This data suggests that CEACAM6 plays a crucial role in the LF82 bacteria uptake. These findings are in line with the previously published studies that have shown that the LPF-negative mutant strain of LF82 failed to interact with murine and human PPs [112]. Additionally it has previously been shown that CD patients have an abnormally high expression of the CEACAM6 receptor on the ileal surface, which makes it easier for the bacteria to bind to and adhere to the enterocytes via this receptor [145, 166]. However, our study is the first to show that LPF plays an important role in the ability of the LF82 bacteria to penetrate the intestinal barrier via FAE and to prove the involvement of the CEACAM6 receptor in the uptake of LF82 bacteria in FAE epithelium and VE of CD patients.

Human α -defensin 5 is a part of the defense mechanism against pathogenic bacteria in the body [167]. Like many other defense systems during CD the production of these antimicrobial peptides is decreased in CD patients [168, 169]. We were curious to study the effects of LF82 bacteria and the human α -defensin in the *in vitro* FAE model and in an *ex vivo* set up on patient material. We hypothesized that LF82 is able to escape the bacteria harmful properties of human α -defensin 5, and that by adding this defensin we would not see an effect on the passage of bacteria. The hypothesis turned out to be wrong. We did in fact observe a significant decrease in the passage of the LF82 bacteria caused by the defensin-exposure in the *in vitro* FAE and VE model as well as in the patient derived FAE tissue in the Ussing chamber. These findings demonstrate that the production of human α -defensin 5 could be a strong defense mechanism against the pathogenic invasion that takes place during CD. It is worth pointing out that the dose of human α -defensin 5 that was used in the present study 0.1Mm was found to be sufficient to reduce the passage of AIEC LF82 through the epithelial monolayer *in vitro* and through tissue specimen *ex vivo*.

Other studies have, however, shown that a concentration between 3.1 μ M [170] to 20 μ M [124] (depending on the experimental set up) is sufficient to guarantee zero survival of *E.coli*. However, in these studies the experimental set up differed from the one that was used in our research since the bacteria were directly exposed to human α -defensin 5 and the cell bacteria interaction process was excluded.

There have recently appeared a number of studies focused on using defensins for therapeutic purposes. For instance, the active region of human α -defensin 5 has been used to create a defensin-derived antibiotic design. These findings lead us to believe that further studies of the effects of human α -defensins 5 are needed and using their structure for therapeutical purposes would be beneficial.

Further studies of defensins' structure and function are of great importance for the discovery novel and efficient antimicrobial drugs.

Concluding remarks

The projects that have been performed within the PhD thesis have combined several aspects of the CD etiology and have given a unique insight into the etiology, mechanisms, and possible potential treatment of CD. The research has shown that FP has beneficial effects on reducing inflammation in a mouse model. It has previously been demonstrated that due to the dysbacteriosis in CD patients the commensal bacteria that do have an anti-inflammatory effect and that help maintain the microflora in the intestine are underrepresented in the gut of CD patients. That is why the fact that we show that an anti-inflammatory commensal strain of bacteria is able to reverse the effects of DSS and improve the mice condition is very important for understanding of the mechanisms behind intestinal inflammation. We further studied the bacterial cell interaction in both *ex vivo* and *in vitro* set up of bacteria that has been isolated from the colon of CD patients. It has been revealed that HM427 used the lipid rafts formation to adhere and penetrate the epithelial cells under the TNF influence. It has already been demonstrated that TNF plays a vital role in promoting constant inflammation in CD patients and disrupting the barrier function, however, we believe that the effects of TNF on the cell-bacteria interaction has been demonstrated for the first time. Moreover, this pro-inflammatory cytokine also might play a role in the bacterial-epithelial cell interaction in the ileum of CD patients. Specifically, it has been shown that the TNF stimulation induced the expression of CEACAM6 receptor in Caco-2 cells [145] thus leading us to believe that high TNF levels do not only lead to barrier disruption due to the interference with TJ proteins and inducing apoptosis of epithelial cells [72] but also induce the expression of the CEACAM6 receptor. Our findings have shown that LF82 bacteria use the CEACAM6 receptor to adhere and penetrate the epithelial barrier in the FAE of CD patients. The possible mechanism could be that LF82 bacteria penetrate the barrier via FAE, thus signaling to the underlying macrophages and triggering pro-inflammatory cytokine production causing constant inflammation and increased colonization in this way. This hypothesis is also supported by the fact that LF82 has the ability to survive and replicate within the macrophages, hence avoiding a natural body immune response as it has previously been shown.

It has also been demonstrated that LF82 does not have the ability to avoid the antimicrobial effects of human α -defensin 5. By adding these antimicrobial peptides into our model we have shown that human α -defensin 5 decreases the passage of LF82 across FAE. The problem, however, remains

that CD patients have a decreased production of human α -defensin 5 which is possibly due to genetic factors.

The findings discovered in the course of these projects covered several etiological issues involved in the development of CD as well as mechanisms behind the bacterial-epithelial cell interaction in CD. These findings offer new possible therapeutic ideas for future treatment of CD (Figure 12).

Specific conclusions

- *Faecalibacterium prausnitzii* supernatant has a positive effect on inflammation in DSS colitis.
- Infliximab treatment restores the mucosal barrier to AIEC in colonic CD. Moreover, AIEC uses the lipid rafts formation to penetrate the epithelial cell barrier under the influence of TNF in an *in vitro* set up.
- AIEC exploits the FAE of CD patients as a portal to penetrate and invade the intestinal epithelium by means of the CEACAM6 receptor in the *ex vivo* and *in vitro* models.
- The uptake of AIEC is reduced by the addition of human α -defensin 5. Furthermore, the expression of human α -defensin 5 is lower in the crypt of the CD patients compared to the non-IBD control patients

Figure 12. A schematic image of the major findings of the projects performed in the course of the PhD thesis preparation.

The schematic image represents the bacteria-cell interaction and the defence mechanisms in a healthy body vs a CD patients body, using FAE as a model. Epithelial and M cells, the expression of the TNF and CEACAM6 receptors as well as the balance between the pro- and anti-inflammatory cytokines secretion are shown in the image.

Acknowledgement

I would like to thank my supervisors, for giving me the opportunity to be part of their research group and to perform these theses.

Åsa Keita, thank you for giving me guidance, introducing me to new techniques and always being available when I had questions. You have always provided me with support and help.

Johan Dabrosin Söderholm, thank you very much for taking me in to your group, giving me the possibility to work with you. Thank you for making time for me in your extremely busy schedule. You are always able to see light in all the results I brought, and most of them looked ‘promising’

I would like to thank **Ylva Braaf**, for all the help I got in the Ussing lab, we really miss you in the lab.

Lena Svensson, you are the one of the nicest people I have met. It has been a great pleasure working with you. Thank you for all your help.

Anders Carlsson, it was so much fun working with you. Thank you for helping me start up in the group, for introducing me to the lab, showing me how to work with animals. I am very happy I got to know you before you left Sweden.

Filipe Meira De Faria, I have not had a chance to work with you, however I did have a chance to have some nice conversations with you. You are such a fun and positive person, I am sure that you will enjoy working in this group.

Elena Vikström, thank you so much for all your support. I am so grateful for everything you taught me and thank you for always being there for me. Good luck with your research!

Vesa Loitto, you have been an absolute life saver for all of my microscopy experiments. I could not have done this without you. Thank you so very much for always being there whenever I had any question.

Ida Schultz, it was a pleasure to meet you and write the paper with you. Thank you for always making time in your busy schedule for all the questions I had.

Mary Esping, you are the fundament of this department, everything would just crash if it weren't for you! Thank you so much for all the hard work you put to keep our department going.

Arthur Wang, thank you very much for all your help.

Derek, Catherine and Fiona-May McKays. I am extremely happy that I got the chance to meet you. Derek, you have completely changed my way of thinking and my attitude to research. I am so happy you came into my life at the point when you did. I am so happy to have had the opportunity to have met your beautiful family, wish we could meet again.

Isabell Olivier, my beautiful French friend! We have shared so many fun times together! You have taught me so much. Everything from how to normalize my data to how to handle mice. I loved hanging out with you at work and outside work, I am so grateful to the universe that we met and that we still remain friends even if we live in different countries.

Martin Tinnerfelt, you are such a great addition to our group, I mean it in the best way possible. Things have been working so much smoother and easier since you joined. Thank you for always being there whenever I needed help or just someone to talk to.

Maite Casado Bedmar, so glad you joined our group. It was so much fun meeting you and having all the discussions.

Stephanie Heil, so glad to have met you, thank you for sharing the office with me as well as 'taking care of me' during our conference in Barcelona.

Kajsa Holmgren Peterson, thank you so much for being there from the very beginning of my scientific journey in Sweden. You have been such a great leader of my Master's program. I am so glad to have been working so close to you all these years.

All my co-workers at Medical Microbiology, thank you for all the good times we shared.

Said, you have always been so supportive and helpful, even if you were not involved in my research, you always cared, I am so grateful for that.

Malin Olsson, you have been a life saver, when it comes to getting our samples. Thank you so much for being so kind and patient with me.

All the personal at the surgery unit, especially: Pär Myrelid, Disa Kalman, Bärbel Jung thank you so much for helping us get all the tissue samples for our studies. This work would not have been possible if it were not for you.

Elin Boström Holm, thank you for all your help with getting the samples and for all the fun conversations we had.

Linda Gerdin, thank you for always bringing light and laughter whenever you entered the room.

Christoffer, thank you so much for all of our fun talks.

Angelica, my dear friend we have known each other for so long and have supported each other through the difficult times and laughed together during the fun times. Thank you for always being there.

Alejandro, I am so happy I got to know you, so grateful for all the fun discussions we had and for all the support you provided for me. Whatever I needed I knew you were always there for me.

Clara, you have been there with me almost from day one. I know I can always turn to you for help and advice. You are one of the nicest people I know, thank you!

Johanna, thank you for all the discussion we had, it was so fun working with you and getting to know you.

Sonja, thank you so much my friend for all the fun we had together. You have been a great support for me.

Nikki, so funny how we met. I am so glad I got to know you! Thank you so much for always being kind and supportive. And a huge thank you for letting me observe the procedures in the hospital. I always get to learn something new from you!

Georgios, it was so much fun working with you, thank you for all the fun conversations we had.

JP and Lina, my dear friends from Örebro. I am so happy to have met you guys, thank you so much for all the fun times and discussions we had.

Our students, especially Mikael, Susana and Julia. You guys have taught me so much by asking all the questions. It was a pleasure working with you. Thank you for your contribution to the projects.

All my amazing friends, whom I met in Sweden during my Master's program. Nina, Sandeep, Sudeep, Nabeel, Jaja, Ellie, Sasha thank you so much for all the good times, support and discussions.

My parents. Dad, if it was not for you I would never have even come to Sweden to study, everything that I have achieved is thanks to you. Thank you for pushing me, telling me there is always room for improvement and never letting me stop and telling me there is always more awaiting☺

Mom, thank you for being there for me always. You are the one who can always calm me down, help me see the positive in any situation or just explain that if there is nothing positive I should just get myself out of it.

Mom and dad, I love you both to the moon and back.

My dear brother **Kirill**, thank you for checking in on me and supporting me when I needed it the most. Especially when I just moved to Linköping. You are the most cold headed person in our family and you always help me cool down. Love you.

Babushka, wish you were here to see me defend my thesis, I miss you every day....

Hazim, my wonderful father in law, thank you so very much for all your support and words of wisdom. You have always cheered me up through all the hard times.

Aidah, my beautiful mother in law! I have been thinking a lot about you when writing my thesis. You are such an inspiration having written your thesis while working and having your three boys. Thank you very much for all your support, help and advice.

Hamme, my love, my support system, my everything! There are no words to describe how much you have helped me to achieve this goal. You have always been the one who told me I can go on, even when I felt that I can't. You gave me strength, you provided me with support and you surrounded me with love. Without you this would not be possible! Thank you!

Sabrina and Oliver, my beautiful angels. You give me strength every day. Thank you for showing me that I can do everything, balance family and work very well. Thank you for sleeping tight so mommy could work at nights, thank you for not getting sick to often. Thank you for the hugs and kisses and smiles that gave me energy to push forward. I love you more than anything!

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