Molecular biosignatures and gut-barrier alterations in inflammatory bowel diseases

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To Nanda

"Somewhere, something incredible
is waiting to be known"

Carl Sagan

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ABSTRACT

Inflammatory bowel diseases (IBD) are chronic immune-mediated disorders affecting the gastrointestinal tract. The multifactorial pathophysiology of IBD is commonly explained as an abnormal interplay between genetic, immune, environmental and microbial factors. However, the understanding of the mechanisms behind IBD pathogenesis is far from complete, which limits the assessment of disease phenotypes and the identification of optimal therapy choices for an effective individualized care.

This thesis has advanced the understanding of disease specific immune features on systemic as well as mucosal level, allowing for improved characterization of disease activity and phenotypes. Analyses of systemic protein profiles and mucosal gene expression identified sustained alterations in Th17 axis and barrier function during active disease and in remission, which suggest that the dysregulation of these mechanisms is involved in the relapse and remitting disease pattern observed in IBD. Further, we demonstrated that the intestinal microenvironment harbors disease specific metabolite profiles and induces distinct effects on epithelial cells *in vitro*. Hence, fecal supernatants, here considered as a proxy for the luminal microenvironment, from patients with colon cancer, IBD and irritable bowel syndrome induced distinct gene expression patterns in intestinal epithelial cell cultures. This indicates that the experimental setup may be an approach to study the crosstalk between the gut epithelium and the luminal content.

In conclusion, the results of this thesis have improved the immunological knowledge of disease activity and phenotypes, thereby guiding future studies of how to treat and prevent flares for optimal individualized therapy. Further, a promising strategy for exploring how the luminal content interacts with the epithelial barrier and contributes to the presentation of disease pathogenesis.

Keywords: Inflammatory bowel diseases, ulcerative colitis, Crohn's disease, immunopathogenesis, IBD.

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SAMMANFATTNING PÅ SVENSKA

Inflammatorisk tarmsjukdom (IBD) karakteriseras av en kronisk inflammation i tarmens slemhinna. Tillståndet anses orsakas av ett dysfunktionellt samspel mellan individens genetiska egenskaper, immunförsvar, tarmbakterier och miljö, där immunsystemet reagerar mot den förmodade normala bakteriefloran. De underliggande mekanismerna för immunopatogenesen vid IBD är ännu långt ifrån klarlagda, vilket försvårar karakteriseringen av den individuella patientens sjukdom samt behov av läkemedelsbehandling. Syftet med denna avhandling var därför att klargöra sambandet mellan systemiska och lokala immunologiska profiler och sjukdomsaktivitet hos patienter med IBD, samt att påvisa effekterna av tarmens lokala mikromiljö för upprätthållande av barriärfunktionen vid olika sjukdomstillstånd som drabbar mag-tarmkanalen.

Analyser av den immunologiska proteinprofilen i blodet samt genuttrycket i tarmslemhinnan visade på dysfunktionell IL17 signalering och barriärfunktion hos patienter med såväl aktiv som inaktiv IBD. Dessa resultat indikerar att en felaktig reglering av nämnda mekanismer är involverad i sjukdomens förlopp med perioder av akuta skov som följs av remission. Avhandlingen visar också att den lokala mikromiljön i tarmen utgörs av en komplex sammansättning av metaboliter som är unik för olika tarmsjukdomar. Epitelceller från tarm odlades *in vitro* i närvaro av fekala supernatanter, ett estimat av tarmens lokala mikromiljö, från patienter med koloncancer, IBD respektive irritabel tarm (IBS). Stimulering av epitelceller med fekala supernatanter från de olika patientgrupperna gav upphov till förändringar i genuttrycket, vilket också skiljde mellan grupperna. Resultaten visar att tillvägagångsättet kan användas för att studera interaktioner mellan tarmens epitelceller och den lokala mikromiljön.

Sammanfattningsvis kan resultaten som beskrivs i denna avhandling förbättra immunologisk karakterisering av sjukdomen och dess aktivitet hos IBD patienter, men kan även utgöra underlag för framtida studier som avser identifiera rätt läkemedelsbehandling för rätt patient. Därutöver beskrivs en lovande strategi för att studera och öka kunskapen om hur tarmens lokala mikromiljö interagerar med epitelcellerna och därmed bidrar till patogenesen vid olika sjukdomstillstånd i mag-tarmkanalen.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Moraes Holst L**, Magnusson MK, Mavroudis G, Polster A, Jonefjäll B, Törnblom H, Sundin J, Simrén M, Strid H, Öhman L. Systemic inflammatory protein profiles distinguish irritable bowel syndrome (IBS) and ulcerative colitis (UC), irrespective of inflammation or IBS-like symptoms

   Inflammatory Bowel Diseases 2020; 26(6): 874–884

II. **Moraes Holst L,** Halfvarson J, Carlson M, Hedin C, Kruse R, Lindqvist CM, Bergemalm D, Almer S, Bresso F, Lundström ML, Repsilber D, D'Amato M, Keita ÅV, Hjortswang H, Söderholm J, Sundin J, Törnblom H, Simrén M, Strid H, Magnusson MK and Öhman L. Downregulated mucosal autophagy, alpha kinase-1 and IL-17 signaling pathways in active and quiescent ulcerative colitis

   Clinical and Experimental Gastroenterology, volume 2022: 15 129–144

III. **Moraes Holst L**, Iribarren C, Sapnara M, Isaksson S, Savolainen O, Wettergren Y, Törnblom H, Strid H, Simrén M, Magnusson MK and Öhman L. Effects of luminal factors on the epithelial barrier in gastrointestinal diseases

   Submitted manuscript

CONTENT

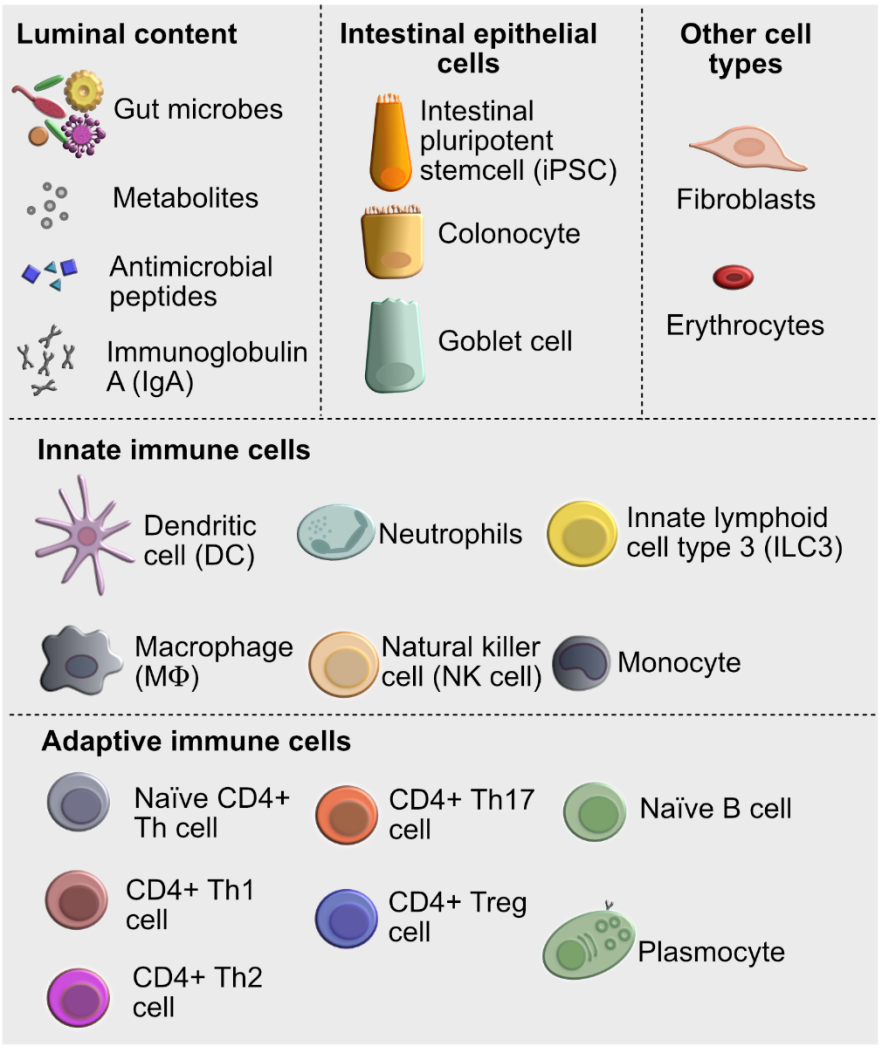
ABBREVIATIONS

Ab	Antibody
ALPK1	Alpha kinase 1
AMP	Antimicrobial peptide
ATG16L1	Autophagy related 16 like 1 protein
AXIN1	Axin-1 protein
CAC	Colitis associated cancer
CARD9	Caspase recruitment domain family member 9
CASP	Caspase
CD	Crohn's disease
CDAI	Crohn's disease activity index
CRC	Colorectal cancer
CRP	C-reactive protein
CXCL	C-X-C motif ligand
DC	Dendritic cell
DSS	Dextran sulfate sodium
FGF21	Fibroblast growth factor 21
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GSRS	Gastrointestinal symptom rating scale
GWAS	Genome wide association study
HBI	Harvey-Bradshaw index
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IBS-SSS	Irritable bowel syndrome severity scoring system
IBS-C	IBS with predominant constipation
IBS-D	IBS with predominant diarrhea
IBS-M	IBS with mixed bowel habits
IBS-U	IBS unsubtyped
IEC	Intestinal epithelial cell
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IL	Interleukin
IL10RA	IL10 receptor subunit alpha
ILC	Innate lymphoid cell
iPSC	Intestinal pluripotent stem cell

IRGM	Immunity-related GTPase family M protein
M cells	Microfold cells
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MΦ	Macrophage
MUC2	Mucin 2
NFκB	Nuclear factor kappa-light chain enhancer of activated B cells
NOD2	Nucleotide binding oligomerization domain containing 2 protein
NOS2	Nitric oxide synthase 2
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
PCA	Principal component analysis
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
RA	Retinoic acid
ROS	Reactive oxygen species
SCFA	Short-chained fatty acids
STAT	Signal transducer and activator of transcription protein
TGFβ	Transforming growth factor beta
Th cells	T-helper cells
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulphonic acid
TNF	Tumor necrosis factor
TNFSF14	Tumor necrosis factor superfamily member 14
Treg	T-regulatory cell
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis

GRAPHICAL DEFINITIONS

Graphical representations of cells and molecules used in this thesis.



1 THE GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract is composed of a series of interconnected organs that are responsible for processing ingested foods and substances for nutrient uptake. Assisted by the adjacent lymphatic, nervous, hepatobiliary and pancreatic systems, it mediates interaction between the host and the external environment. It is anatomically divided in two major parts: the upper GI tract, including the oropharynx, esophagus, stomach and duodenum, and the lower GI tract including the jejunum, ileum, colon and rectum.

The leading defense mechanism in the GI tract is the specialized mucosal tissue, more specifically the epithelial barrier that covers all of its surfaces and serves as an interface between the host and the environment. The GI tract holds the largest mucosal surface in the human body, constantly exposed to different antigens and the microbiome, hence its critical role in immune regulation.[1] It is responsible for the vital task of maintaining immune tolerance to dietary antigens and commensal bacteria while protecting the host from potential pathogens.[1]

Alongside the variation in function regarding food digestion throughout the GI tract, the structure as well as the physiological role of the epithelium also varies. In some sites, such as the esophagus, the epithelial barrier is composed of multiple layers of non-keratinized stratified squamous cells to handle the peristalsis of larger pieces of food. In contrast, the lower GI tract comprises of a single layer of columnar cells, thin enough to enable nutrient absorption and the sensing of the environmental stimuli. The single cell epithelial barrier is supplemented with a large variety of molecules and mechanisms of defense, such as the mucus layer, antimicrobial peptides (AMPs) and antibodies.

1.1 THE COLONIC MUCOSAL IMMUNE SYSTEM

In the GI mucosa, different cell types participate in the multiple cellular interactions that sustain homeostasis and respond to harm. Thus, the successful resolution of acute injury or maintenance of gut homeostasis is a group effort that depend on a finely orchestrated communication

between the epithelial barrier, the lamina propria and the gut-associated lymphoid tissue (GALT).

Anatomically, the mucosal immune system is organized in two parts based on their function, the inductive sites and the effector sites.[2] The inductive sites consist of the GALT, including isolated lymphoid follicles, scattered colonic patches and mesenteric lymph nodes.[3,4] There, antigen-presenting cells (APCs), such as dendritic cells (DCs), sense the luminal environment by sampling antigens that reach the mucosal surface. Later, DCs activate B and T lymphocytes, which in turn migrate to effector sites, composed by the lamina propria and the intestinal epithelium, where they will exert their effector functions.

In its task to maintain a balanced relationship with the luminal environment, the colonic mucosa sustains a state of "physiological inflammation", including innate and adaptive immune responses. The complex modulation of these responses begin at birth and continue to evolve throughout life, following diet, environmental and health changes.[5] Competent protective immunity against pathogens while preserving tolerance against commensal microbiota and food antigens is essential to prevent the dysfunctional inflammation observed in inflammatory bowel diseases (IBD).[6]

1.1.1 COLONIC EPITHELIAL BARRIER

The colonic epithelial barrier is the first line of defense against the luminal environment. Most of the colonic epithelium is organized in tightly juxtaposed crypts, called crypts of Lieberkühn, which are invaginations of the mucosa that host intestinal pluripotent stem cells (iPSCs) at its base as illustrated in figure 1.[7] It is composed by a single lining of polarized and mixed intestinal epithelial cells (IECs) that are continuously replenished by the iPSCs.[7]

There are mainly two different types of IECs in the colonic epithelium: colonocytes and goblet cells.[8] Other cell types, such as enteroendocrine cells, Tuft cells and microfold (M) cells are scarcely distributed in the colon tissue and represent less than 1% each of all specialized IECs.[9,10] The most prevalent one is the colonocyte, characterized by its well-defined polarization and complex brush border, with an absorptive function (Figure 1). Colonocytes are valuable sensors of the luminal content and important players in the innate immune system. Their maturation is closely dependent on microbial metabolites, such as short chain fatty acids (SCFAs), which grants them critical dependence on the

microbial balance.[11] They are tightly connected through tight junctions, comprised of complexes of proteins from the occludin and the claudin families, whose function is to control the communication between the host and the lumen, filtering the passage of fluid and metabolites.[12]

Goblet cells are secretory IECs and the second most abundant cell type in the colon.[8] Their main function is to secrete mucins that form the thick mucus layer covering the colonic epithelium, which protects it from mechanical stress and the luminal bacteria. Among the different types of mucins secreted by the goblet cells, the most abundant is mucin-2 (MUC2), which forms the compact inner mucus layer.[13] Proteolytic cleavage of MUC2 by anaerobic mucus-associated bacteria at the luminal surface of the inner mucus layer is responsible for the generation of the outer mucus layer.[14]

Jointly, IECs and the mucus layer, constitutes the interface between the host and the luminal content, mediating the gut-barrier crosstalk (Figure 1). Non-pathogenic microbes or antigens that breach the mucus layer can be taken up by colonocytes and recognized by their pattern recognition receptors (PRRs), triggering secretion of cytokines, such as transforming growth factor beta (TGFβ) and thymic stromal lymphopoientin (TSLP).[15] Synergistic secretion of TGFβ and TSLP is important for inducing a tolerogenic phenotype in DCs regulating inflammatory responses (Figure 1).[16] Following cytokine stimulation, DCs secrete interleukin (IL)23, which induces AMP secretion by IECs, through IL22 signaling, secreted by macrophages (MΦ), innate lymphoid cells (ILCs) and natural killer (NK) cells. AMPs diffuse into the mucus layer and act in the lumen, controlling the microbial population (Figure 1). Alternatively, upon direct invasion and epithelial damage, PRR signaling in IECs will activate the transcription factor nuclear factor kB resulting in secretion of pro-inflammatory cytokines including tumor necrosis factor (TNF), IL6, C-X-C motif chemokine ligand (CXCL)8 and IL18, which will initiate an inflammatory response in the lamina propria.[17]

1.1.2 LAMINA PROPRIA

The lamina propria consists of the loose connective tissue below epithelial surface that permeates the spaces between colonic crypts. It is the main effector site, hosting a myriad of effector immune cells.[18] There, resident phagocytes (DCs and MΦ) work continuously to sample and clear the mucosa by engulfing microbes and degrading them into antigens that will be presented to other specialized immune cells.

Further, stromal cells, such as fibroblasts, act synergistically with MΦ in the lamina propria to respond to harm and resolve inflammation.[19] Cells from the lymphoid lineage, including activated CD4+ T helper (Th) cells and CD8+ cytotoxic T cells, immunoglobulin (Ig)A-secreting plasma cells, non-conventional T cells and ILC are also abundant in the lamina propria and essential for the mucosal immune response in the gut (Figure 1).[20]

The cell-cell and cytokine mediated interactions in the lamina propria sustain a tolerogenic environment. Activated IgA-secreting plasma cells and T cells originate from the inductive sites where naïve B and T cells differentiate after cognate interaction with antigen bearing major histocompatibility complex (MHC) class I or II on APCs. CD4+Th cells undergo differentiation into the appropriate Th subset depending on the cytokine signals in the inductive site.[21] Following maturation, differentiated lymphocytes upregulate gut-homing integrin $\alpha_4\beta_7$ that allows them to migrate to the gut mucosa.[22] Once in the lamina propria, IgA-secreting plasma cells sustain antibody (Ab) secretion, while CD4+ Th cells are activated and begin secreting their signature cytokines as shown in figure 1.[23,24]

The colonic lamina propria is rich in CD4+ T cells and a proper balance between the various subsets is crucial for homeostasis and competent response to injury. At steady state, T regulatory (Treg) cells are predominant, secreting anti-inflammatory cytokines IL10 and TGFβ (Figure 1). Local secretion of TGFβ by the epithelial barrier, phagocytes and stromal cells, in combination with retinoic acid (RA) and luminal production of SCFAs, contribute to peripheral differentiation of the Treg phenotype that withholds abnormal immune reaction to commensal bacteria and food antigens.[25]

The first described CD4+ Th subsets were the interferon-γ (IFNγ) secreting Th1 cells, responding to intracellular microbes, and the IL4, IL5 and IL13 secreting Th2 cells, responding to parasites and participating in allergy responses. Even though both subsets are linked to pro-inflammatory immune responses, their steady and controlled presence in the tissue is necessary due to their protective function against external harm. Polarization towards Th1 or Th2 type responses was initially described as mutually exclusive, since one is able to suppress the differentiation of the other.[26,27] However, newer evidence suggests a certain level of plasticity in these cells that allows them to shift between phenotypes depending on local signals.[28]

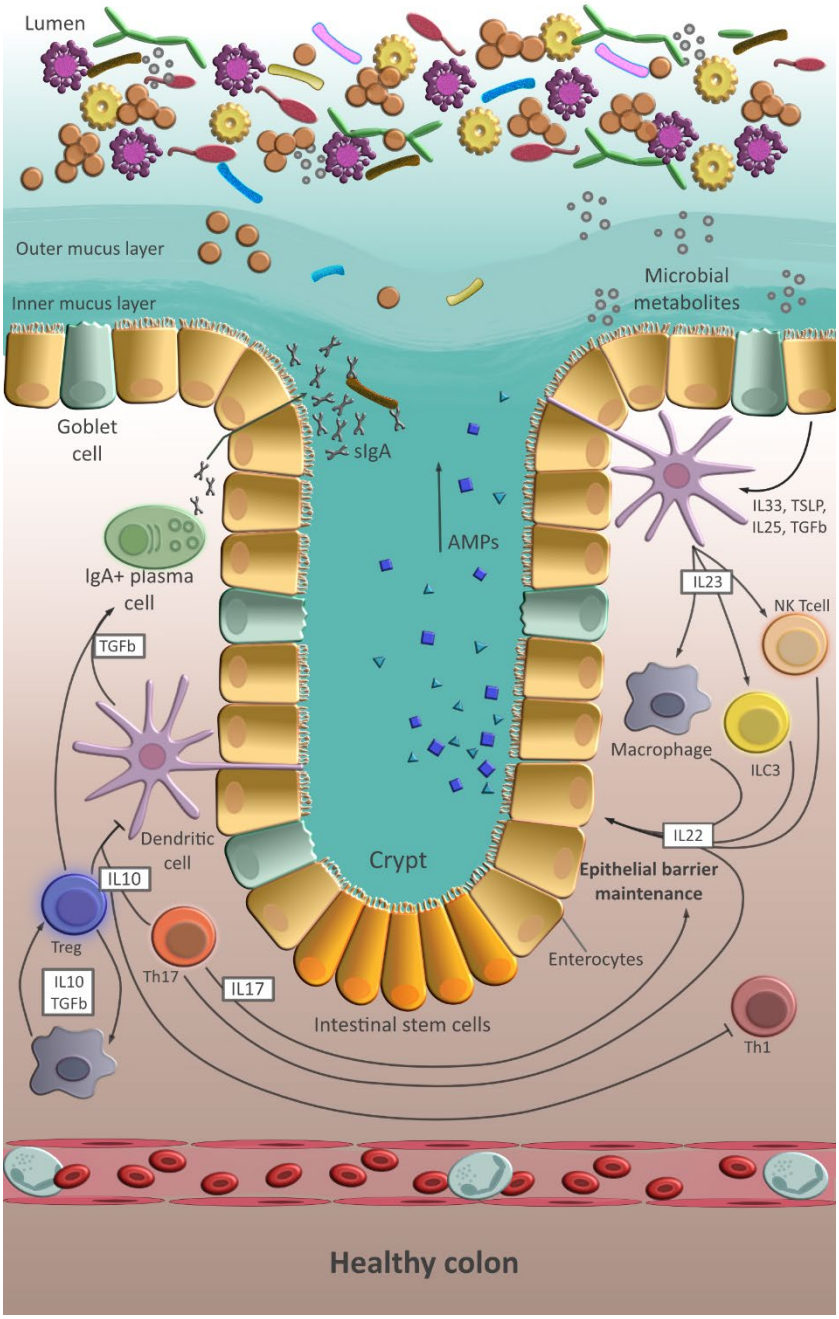


Figure 1. Schematic representation of the cell interactions in the colonic mucosa during homeostasis. → refers to stimulation and ┤refers to suppression

Stepping away from the classic Th1-Th2 paradigm, other Th subsets are also of major importance in the healthy colonic lamina propria. Particular interest is given to IL17 secreting Th17 cells. Th cells acquire a Th17 phenotype in the presence of IL6, IL23 and TGFβ and secrete IL17 cytokines, which mediate the immune responses against extracellular microorganisms.[29] However, Th17 cells seem to present a plasticity of their own that direct either towards anti- or pro-inflammatory functions. In face of a tolerant environment rich in TGFβ, they preferably secrete IL22 and IL10 (Figure 1).[30] In contrast, high levels of pro-inflammatory cytokines IL1β and IL6, shifts them into an inflammatory phenotype, secreting IL17A and IL17F.[31]

1.2 COLONIC LUMINAL MICROENVIRONMENT

The GI tract harbors the largest microbial community in the human body, and the colon is where the greatest diversity of microbes reside.[32] Even though research has focused on describing bacterial species, archaea, viruses, parasites and fungi are also part of the commensal and/or symbiotic ecosystem that is referred to as gut microbiota.[33] In a context of health, microbes perform many beneficial functions, such as vitamin synthesis, nutrient digestion and fermentation that contribute to the health of the human host.[34] In turn, humans provide shelter, a diversity of aerobic and anaerobic niches, and food for microbial growth and survival. Changes in diet, habits and environment, as well as infections and medications, can have direct impact on nutrient resources and create a selection pressure that molds the microbial diversity, and affects health.

During early childhood, environmental factors such as delivery mode, use of antibiotics and breastfeeding, influence the establishment of the gut microbiota. Maturation of the immune system is closely dependent on early colonization of the gut with specific microbes, such as *Bifidobacterium spp* and *Bacteroides,* which produce SCFAs and act as important modulators of the immune response in the mucosa and systemically.[35] Maintenance of gut homeostasis by luminal microbiota is a reciprocal process. Early breastfeeding shapes the microbiota by selectively feeding SCFA-producing bacteria and neutralizing pathogens through IgA present in the human breastmilk.[36] In turn, SCFA and vitamin A-derived RA induce differentiation of Tregs and Th17 cells, which suppress inflammatory responses towards the newly established gut microbiota and stimulate secretion of AMPs and mucus,

further controlling microbial growth.[25] Throughout development, ingestion of dietary fibers continues to support SCFA-producing bacteria and is believed to contribute to health.

Apart from the gut microbiota, the luminal microenvironment carries a great amount of other molecules and chemical compounds that reflect dietary intake, environmental exposures, physiological host secretions and microbial metabolism.[37] These compounds are jointly called metabolites and exert biological effects in the gut. Microbial-derived metabolites, such as SCFAs, are often a result of the resources available in the lumen and seem to be more sensitive to environmental changes than the microbiota diversity. They can therefore be considered as a dynamic imprint of the present luminal environment, host habits and a translation of the microbial activity rather than fluctuations of microbial diversity.

2 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a chronic immune-mediated disorder of the GI tract typically divided into two major subtypes, Crohn's disease (CD) and ulcerative colitis (UC).[38] IBD commonly arises in early adulthood and evolves with alternating phases of disease activity and clinical remission as a result of chronic inflammation.[39] As a life-long disease, IBD imposes a high societal burden apart from the repercussions in the everyday life of the patient.[40]

Traditionally, IBD has been described as a western disease, with higher prevalence in Northern Europe and North America and empirically associated with the profound changes in lifestyle and living conditions observed throughout the past century.[41] Indeed, increasing prevalence of UC and CD in developing countries in parallel with the incorporation of westernized lifestyle and dietary habits suggests that the environment and lifestyle play an important role in the pathogenesis of the disease.[42] Further evidence supporting the role of the environment in IBD is seen in children migrating from areas of low prevalence to areas with high prevalence of IBD, who assume a similar risk of developing the disease as the population residing at the new site of residence.[43]

Although UC and CD share common clinical features such as bloody diarrhea, weight loss, anemia and fever, the characteristics of the mucosal inflammation are rather distinct. Typically, CD is characterized by its segmental lesions of transmural inflammation, which may affect any part of the GI tract, although more common in the terminal ileum.[44] In contrast, UC affects the colorectal mucosa and submucosa, with lesions in the rectum that extend continuously towards more proximal parts of the colon, with different extent of inflammation among patients (Table 1).[45]

Table 1. Clinical characteristics of Crohn's disease and ulcerative colitis

	Crohn's disease	**ulcerative colitis**
Location	Entire GI tract (most common in the ileum and colon)	Limited to the colon
Macroscopic findings	Skip lesions Fistulas Strictures	Continuous lesion starting in rectum No fistulas or strictures
Histologic findings	Discontinuous and focal crypt alterations Mucosal granulomas Transmural inflammation	Decreased crypt density Diffuse increased cellularity No granulomas Inflammation limited to the mucosa and submucosa

IBD is a prototypical multifactorial complex illness where combined triggers seem to challenge intestinal homeostasis to a tipping point, initiating chronic inflammation.[46] Current evidence suggests that genetic variants, luminal stimuli and dysfunctional immune responses compose this triad of triggers behind the pathogenesis of IBD.[47] Several mechanisms have been proposed to explain the abnormal interplay between these triggers, including decreased microbial diversity and dysfunctional pattern recognition receptor signaling, resulting in an aberrant expression of pro- and anti-inflammatory molecules regulating the immune response, which will be discussed in more detail later in this thesis.

This thesis will focus primarily on the role of the colonic mucosa in the immunopathogenesis of IBD with regards to three different aspects (for graphical overview see Figure 2):

1) Systemic inflammatory protein levels, reflecting disease activity and functional bowel symptoms in patients with ulcerative colitis and irritable bowel syndrome.
2) Host immune response in the epithelial barrier during active disease and remission, investigating possible pathways involved in inflammatory bowel disease pathogenesis.
3) The effects of the luminal content on the colonic epithelial barrier in gastrointestinal diseases, including IBD.

2.1 METHODOLOGICAL APPROACHES TO UNDERSTAND IBD

Due to its multifactorial nature, the scientific approach to understand IBD as a complex disease has changed throughout the years. For many years, oversimplified modelling strategies, focusing on single variables or pathways, have been used in the attempt to understand how isolated factors may contribute to disease. However, translation of these unidimensional findings into patient care has been rather limited, as they do not reflect all the components of a complex system. Indeed, even diseases that are caused by single genes and follow a Mendelian pattern of inheritance display a certain phenotype variability, due to the individuality of the interaction between genetics and environment. Therefore, a multidimensional view at the factors involved in the pathogenesis of IBD is necessary to understand disease phenotypes and improve individualized care.

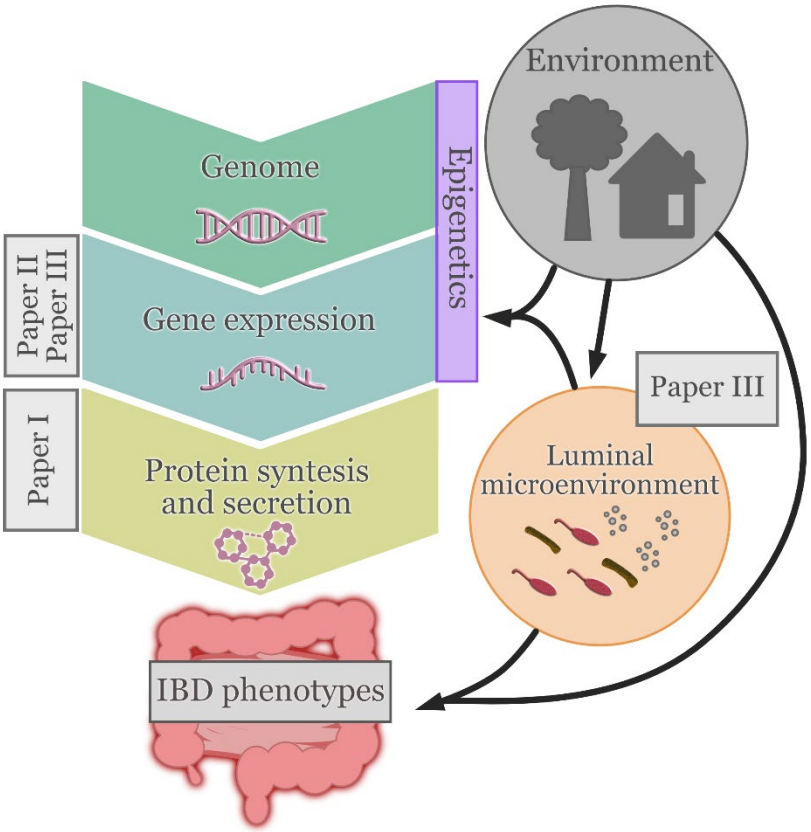


Figure 2. Graphical summary of the factors involved in IBD pathogenesis highlighting main aspects investigated in this thesis.

While the inherited genome is a rather fixed component, the other interacting factors, such as the transcriptome, the proteome, metabolome and microbiome, are diverse and dynamic.[48] Building an illustrative analogy, we could understand IBD as an infinity puzzle, where the starting point is not defined and each piece of the puzzle is able to fit into several other pieces providing multiple possibilities for a final picture. Ideally, science will predict how the puzzle falls into place in order to approach correctly the needs of the individual patient. An option for better understanding of the "IBD puzzle" is to evaluate multiple variables simultaneously and search for patterns or profiles among patient groups, which characterize them according to their phenotypes. Throughout this thesis, several methods, panels and multivariate statistical analysis have been used in the attempt to understand the patterns that characterize IBD phenotypes with regards

to different components of the IBD puzzle (Figure 2): secreted proteins (paper I), gene expression (papers II and III) and luminal microenvironment (paper III).

When it comes to strategies for analysing multivariate data, principal component analysis (PCA) was the main tool of choice in papers I, II and III. PCA is an unsupervised method that reduces the number of dimensions of large datasets, while trying to preserve as much information as possible.[49] As an unsupervised method, PCAs can successfully cluster the observations according to patterns within the data that explain their dispersion in the plot, and even reveal non-intended biases. However, PCAs can fail to identify which variables are most important when differentiating two groups.

To identify the best association between variables and selected patient groups, a supervised method was used in papers I and III, orthogonal projections to latent structures–discriminant analysis (OPLS-DA). In an OPLS-DA, the variables are ranked according to their importance in differentiating the selected observation groups, which allows identification of the relevant parameters that best define each group.[50] A drawback of this method is that it is biased, as it "forces" a separation between the study groups. Therefore, confirmation of the results using post-hoc analysis and validation of the relevant variables in separate cohorts is important.

In paper I, we proposed a characterization of disease-specific protein signatures to understand the potential link between inflammation and irritable bowel syndrome (IBS) symptoms in patients with UC and IBS. For this study, serum levels of 92 inflammation-related proteins were measured by proximity extension immunoassay, using the Proseek Multiplex Inflammation panel (Olink Proteomics, Uppsala, Sweden), which is is a multiplex immunoassay, using magnetic oligonucleotide-labelled antibodies for measuring levels of multiple proteins simultaneously in biological samples.[51] Multiplex protein analysis is a useful tool when investigating the result of gene expression at a given time and context. Moreover, since secreted proteins are not limited to the intracellular compartment, they may diffuse and reach the blood circulation, which allows the measurement of protein levels in serum samples as well as in the mucosa. However, in paper I, protein levels were only measured in serum samples, to investigate the possible systemic profiles defining different disease phenotypes. Integrated analysis of mucosal and systemic protein levels could give a more

complete picture of molecular interactions during disease activity, remission and in the occurrence of functional bowel symptoms, i.e., symptoms compatible with IBS.

In papers II and III, mucosal gene expression was the main focus. For each study, distinct methods for measuring transcriptional data were used. While in paper II, gene expression was assessed using the nCounter Human Host Response panel from NanoString Technologies, Inc, in paper III, gene expression was measured using a custom RT^2 PCR Array (Qiagen). The nCounter Human Host Response panel includes 776 genes associated with five functional themes (host susceptibility, interferon response, innate immune cell activation, adaptive immune response and homeostasis). The large number of targeted transcripts allowed the identification of pathways affected by differentially expressed genes, which suggests the dysfunctional processes possibly contributing to disease immunopathogenesis. In contrast, gene expression in paper III was evaluated using a selected custom array, measuring 87 genes expressed by IECs and representing pro-inflammatory response, antimicrobial response, metabolite sensing, carcinogenesis and cancer immunity, epithelial barrier integrity and a separate group of miscellaneous genes. Since the study was performed using *in vitro* models representing the epithelial barrier, it was relevant to select genes that were known to be expressed by IECs.

A less biased overview of affected pathways and/or genes could be achieved using bulk RNA sequencing instead, where total extracted RNA is examined and may be assessed for differentially expressed genes and pathways. While RNA sequencing may uncover unexpected pathways possibly involved in disease pathogenesis, that were not previously regarded as relevant, it gives space for over-interpretation of the results, as the amount of generated data is overly large. Targeted analyses, limited to immune response, may not be as widely exploratory, but include sufficiently relevant genes and representative pathways to understand alterations associated with disease.

The last component of IBD pathogenesis included in this thesis was the luminal microenvironment, discussed in paper III. In this study, we aimed to establish the effects of patient-derived fecal supernatants, potentially reflecting the luminal microenvironment of the donor of the fecal sample, on *in vitro* set-ups simulating the epithelial barrier. Most published studies evaluate the microbiota and the mucosal immune response separately, which limits the possible conclusions regarding

direct effects of the luminal content on the epithelial barrier. Thus, integrated methods to understand the crosstalk between the luminal content and the host are needed.

2.2 DISEASE HETEROGENEITY IN IBD

In spite of the seemingly clear division between UC and CD, the heterogeneity of the clinical presentation, immune characteristics, genetic factors and microbiota profiles suggest that the variety of phenotypes within IBD is better explained as a disease spectrum rather than classified as two single entities. When it comes to clinical presentation, the natural history of the disease is not linear for all patients. While some experience a slowly progressive disease, others may present with severe and frequent flares at the onset of disease with mild infrequent flares later in life. Further, studies show a cumulative incidence of 18% for colorectal cancer (CRC) in patients with left-sided or extensive colitis, which seems to present a poorer prognosis when compared to non-IBD CRC patients.[52,53]

In addition to an unpredictable disease course, periods of remission are not necessarily asymptomatic. Irritable bowel syndrome (IBS)-like symptoms, such as altered bowel habit, abdominal pain and bloating, have consistently been found to be increased in patients with IBD in comparison with non-IBD controls even in those classified to be in remission, i.e. without signs of active inflammation.[54] Differentiating between IBS-like symptoms and an upcoming flare in IBD patients may be difficult, which can misguide choice of treatment, increase morbidity and negatively affect quality of life. The similarity of the symptoms among the two diseases suggest an at least partly shared underlying immunopathology that would range from low-grade immune activity to full-scale inflammation.

The effects of the chronic inflammation caused by IBD are not limited to the gut. Epidemiological studies report the prevalence of extraintestinal manifestations to range between 19 to 40%, depending on how they are defined.[55-57] Extraintestinal manifestations can affect a large variety of organs including the joints, skin, eyes and liver, and their occurrence can be associated with flare-ups of the intestinal disease.[58] Among extraintestinal manifestations, primary sclerosing cholangitis is an important immune-related manifestation, associated with a substantial risk for colitis-associated cancer.[59] Moreover, it has been proposed that a subgroup of UC patients with co-existing primary

sclerosing cholangitis could compose an unique phenotype that includes more severe inflammation of the right colon, backwash ileitis and apparent sparing of the rectum mucosa.[60] Independent on disease course, CD and UC are dynamic diseases that require continuous assessment to guarantee proper care.

2.2.1 ULCERATIVE COLITIS

For UC, the disease can be characterized with regards to the extent of the intestinal inflammation and disease activity. The most commonly used tool to classify disease extent is the Montreal classification, which divides colon involvement in three categories: proctitis (E1), left side colitis (E2) and extensive colitis (E3) (Figure 3).[61]

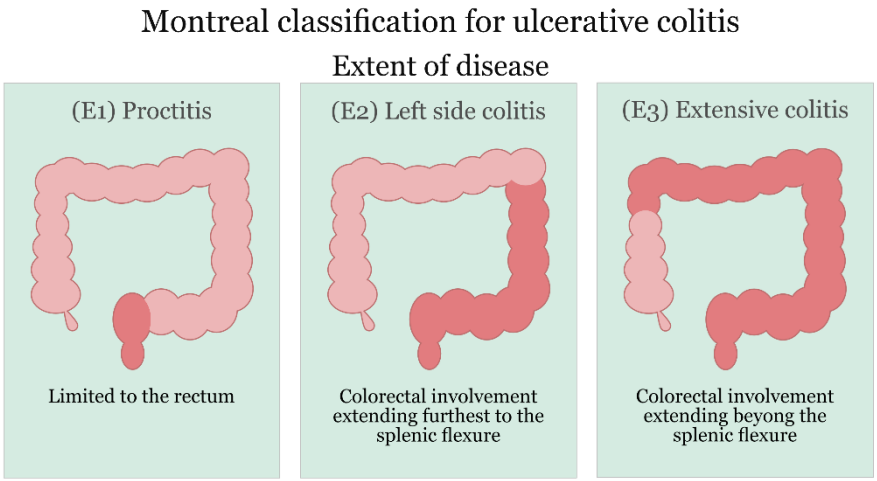


Figure 3. Montreal classification for ulcerative colitis describing the extent of disease. Ulcerative colitis can be classified in three different categories: E1, proctitis; E2, left-side colitis; E3, extensive colitis.

Regarding disease activity, different tools have been described in the literature, combining clinical parameters, patient reported outcome measures and the physician's assessment. Among them, the Mayo score described in table 2 has been used for assessing disease activity in our studies (Papers I, II and III). Others have discussed that the subjectivity of the physician's assessment and the endoscopic score may introduce bias and sway its precision. However, its good correlation with UC activity, reflecting both improvement and worsening of disease, has rendered the Mayo score as tool often chosen as an indirect biomarker of disease activity.[62]

Table 2. Mayo score for disease activity in ulcerative colitis.

Mayo score description	
Stool frequency	
0 =	Normal
1 =	1-2 stools/day above normal
2 =	3-4 stools/day above normal
3 =	>4 stools/day above normal
Rectal bleeding	
0 =	None
1 =	Visible blood in the stools less than half of the time
2 =	Visible blood in the stools more than half of the time
3 =	Visible blood in the stools more than half of the time AND passing blood alone at least one time
Physician's global assessment	
0 =	Remission
1 =	Mild disease
2 =	Moderate disease
3 =	Severe disease
Mucosal appearance at endoscopy*	
0 =	Normal or inactive disease
1 =	Mild inflammation (erythema, decreased vascular pattern, mild friability)
2 =	Moderate inflammation (marked erythema, absent vascular pattern, friability, erosions)
3=	Severe inflammation (spontaneous bleeding, ulceration)
Cumulative score	**Corresponding disease activity thresholds[63]**
≤2	Remission
3-5	Mild disease
6-10	Moderate disease
11-12	Severe disease

*Not included in partial Mayo score

2.2.2 CROHN'S DISEASE

For CD patients, the clinical presentation can differ greatly between individuals.[44] Almost 60% of the CD patients display ileal involvement, including isolated ileitis or ileocolitis, while 30% of the patients present with isolated colitis. Similar to UC, CD can also be classified according to the Montreal classification, which specifies disease location (L) and behaviour (B) (Figure 4).[61]

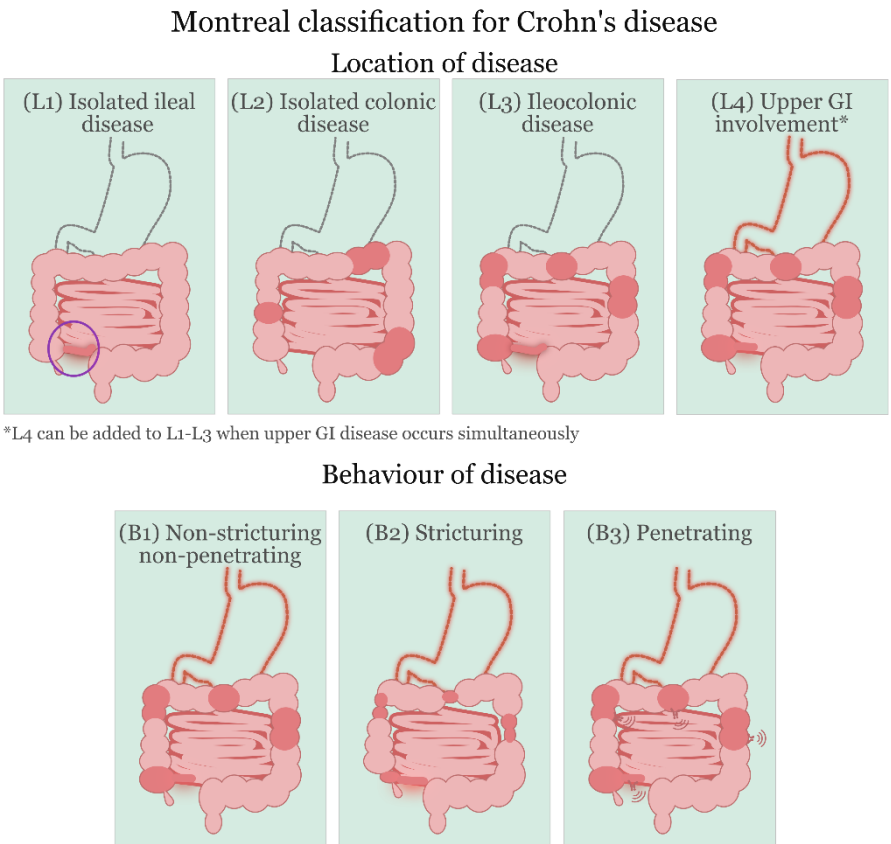


Figure 4. Montreal classification for Crohn's disease, categorized according to disease location (top row) and disease behaviour (bottom row).

Further assessment of disease activity in CD is not as straight forward as for UC, due to the poorer correlation between symptoms and disease severity. The most accepted tool for assessing disease activity is the Crohn's disease activity index (CDAI), which is based on 8 independent clinical variables that generates a score ranging from 0 to ~600 (table

3)[64]. It is, however, a cumbersome tool due to the comprehensive design, depending on a 7-day patient diary for complete scoring, but still extensively used in research studies. A simplified version of the CDAI, called the Harvey-Bradshaw index (HBI), which excludes the patient diary, body weight and haematocrit measures, is considered a simpler tool for the assessment of disease activity and has been used for CD patients in our study (Paper II). Studies show that there is a reasonable correlation between score variation of the HBI and the CDAI, confirming the value of HBI for standardizing clinical assessment in research.

Table 3. Comparison between the Crohn's disease activity index (CDAI) and the Harvey-Bradshaw index (HBI) for assessing disease activity in Crohn's disease.

Variable	CDAI* score definition	HBI† score definition
Total number of liquid or soft stools	2 x Total number of liquid or soft stools in 7 days	Total number of liquid or soft stools in 1 day 0 = 0-1 stools 1= 2-3 stools 2= 4-5 stools 3= 6-7 stools 4= 8-9 stools 5= 10 or more
Abdominal pain	7 x Average rating in the past 7 days: 0= none 1= mild 2= moderate 3= severe	0= none 1= mild 2= moderate 3= severe
General well being	7 x Average daily rating in the past 7 days: 0= generally well 1= slightly under par 2= poor 3= very poor 4= terrible	0= generally well 1= slightly under par 2= poor 3= very poor 4= terrible
Abdominal mass	10 x Rating below: 0= none 2= questionable	0= none 1= dubious 2= definite

	5= definite presence of abdominal mass	3= tender
Complications	One point added to each extraintestinal finding/ complication - Arthritis/arthralgia - Uveitis/Iris inflammation - Erythema nodosum/Pyoderma gangrenousm/aphtous stomatitis - Anal fissure/fistula/abscess - External fistula - Fever>37.8°C in the past week	One point added for each complication - Arthralgia - Uveitis - Erythema nodosum - Aphtous ulcers - Pyoderma gangrenosum - Anal fissures - New fistulas - Abscesses
Use of anti-diarrheic medication	30 x Rating below 0= No 1= Yes	NA
Weight	1 x Percentage deviation from ideal weight	NA
Hematocrit	6 x Percentage deviation from normal hematocrit (47% in men and 42% in women)	NA

Cumulative score	**CDAI**	**HBI**	
<150	Remission	≤4	Remission
150-219	Mild disease	5-7	Mild disease
220-450	Moderate disease	8-16	Moderate disease
>450	Severe disease	>16	Severe disease

**CDAI, Crohn's disease activity index*

†HBI, Harvey-Bradshaw index

NA, non-applicable

2.2.3 BIOMARKERS

Biomarkers are molecules that are measurable in biological specimens and signalize physiological or pathological processes. They are useful tools to guide clinicians in diagnosis, prognosis and prediction of clinical and therapy outcomes.[65] However, few non-invasive, objective, cost-effective, disease specific and easy to perform biomarkers are available for IBD and research in this field has expanded in the past decades. Therefore, the use of biomarkers in clinical practice is still limited to a few serological and fecal markers for the assessment of disease activity.

Among serum biomarkers, C-reactive protein (CRP) is a well-established biological marker of inflammation in IBD.[66] CRP is an acute phase protein that peaks and declines rather quickly in parallel with onset and resolution of inflammation in general. Despite its tight association with active inflammation, the use of CRP on its own for disease surveillance in IBD is not ideal, as patients with mild to moderate mucosal activity may not present increased CRP levels.[67] Still, increased CRP levels are not disease specific and may also reflect presence of an infection elsewhere. However, extensive mucosal involvement is well correlated with CRP levels in IBD.

In the absence of endoscopic assessment, fecal calprotectin can be used as a surrogate marker for mucosal inflammation in the colon. Calprotectin is a calcium-binding protein, secreted by neutrophils that inhibits metalloproteinases and exert antimicrobial activities. Levels >250μg/g are strongly associated with inflammation in the colon and rectum and indicate active disease.[68] Moreover, fecal calprotectin has been found to be superior to CRP in monitoring disease activity,[69-71] with several studies suggesting that its increase precedes flares.[72-74] Fecal calprotectin levels were used in papers I, II and III in combination with the endoscopic assessment, or as a surrogate marker in the absence of endoscopy, to determine disease activity. However, calprotectin is not a disease specific biomarker, since it is also elevated during intestinal infections[75]. As an isolated marker, it is not sufficient for disease diagnosis, but may be used to follow likelihood of disease activity in patients with diagnosed IBD.[76]

2.3 IBS AND IBS-LIKE SYMPTOMS IN IBD

IBS is a functional disorder of the GI tract, characterized by recurrent abdominal pain associated with altered stool frequency and/or consistency.[77,78] The worldwide prevalence of IBS is estimated to be approximately 4% according to recent multicentre studies based on Rome IV criteria,[79,80] and women are more frequently affected than men.[80] In contrast to IBD, where evidence for inflammatory activity and organ damage exists, symptoms in IBS cannot be explained by clear organic causes. Nevertheless, recurrent bowel symptoms greatly affects the quality of life of the patients, negatively influencing their social and work activities.[81]

There are yet no objective biomarkers for IBS. Instead, clinical assessment of the patients is necessary for diagnosis. Laboratory markers that allow exclusion of organic causes for the symptoms, such as CRP, fecal calprotectin and anti-tissue transglutaminase, may have an important role in the initial evaluation. However, confirmation of the diagnosis using a positive diagnostic strategy based on clinical criteria is sufficient.[82,83] Rome IV is the latest diagnostic criteria for IBS,[78] which is defined as a functional bowel disorder with recurrent abdominal pain at least 1 day/week in the last 3 months, associated with at least two of the following criteria:

- Related to defecation
- Associated with a change in stool frequency
- Associated with a change in stool form

Patients included in our studies (Paper I and III) were diagnosed according to Rome III criteria instead, which was defined as recurrent abdominal pain or discomfort, for at least 3 days/month in the past 3 months, associated with at least 2 of the following criteria:

- Improvement with defecation
- Onset associated with a change in stool frequency
- Onset associated with a change in stool form

Studies have shown a higher incidence of IBS when Rome III was used in comparison with the stricter Rome IV criteria.[84] Nevertheless, considering that the patients selected for our cohorts were included with moderate to severe disease, it is most likely they would also fulfil

Rome IV criteria, as patients meeting the Rome IV but not Rome III criteria constitute a more severe group of patients.[82]

In addition to the Rome criteria, IBS patients are subtyped according to predominant symptoms and later classified with regards to disease severity. There are four IBS subtypes, defined according to the characteristics of the abnormal stool forms: predominant diarrhea (IBS-D), predominant constipation (IBS-C), mixed bowel habits (IBS-M) and unsubtyped (IBS-U). Stool forms are defined according to the Bristol stool form scale,[85] where patients identify the appearance of their stools in a scale from one to seven, where one and two are associated with constipation and six and seven with diarrhea. This studies in this thesis (Papers I and III), included only patients with IBS-D and IBS-M, with the intention to compare them to patients with IBD regarding immune mechanisms involved in disease pathogenesis.

The severity of IBS can be assessed by different symptom scales, such as the gastrointestinal symptom rating scale (GSRS) or the IBS severity scoring system (IBS-SSS). While the GSRS evaluates five domains (pain, bloating, constipation, diarrhea and satiety) using a seven-point Likert scale,[86] IBS-SSS is a five-item questionnaire evaluating abdominal pain, distension, bowel dissatisfaction and the interference of symptoms with daily life.[87] IBS patients in our cohorts in papers I and III were evaluated using the IBS-SSS score, which generates a score between 0 to 500 and classifies IBS patients into three groups according to severity: <175 for mild IBS, 175-299 for moderate IBS and ≥300 for severe IBS. For these studies, only patients with moderate to severe disease were included.

The precise cause of IBS is currently unknown. It is considered a multifactorial disease and often explained as a disorder of the gut-brain axis, where central nervous system mechanisms communicate with the enteric nervous system, affecting immune activation, motility and visceral sensitivity.[88] High prevalence of anxiety and depression disorders in IBS patients is well established,[89] supporting the involvement of the gut-brain axis in disease pathogenesis. However, abnormalities in peripheral factors, such as the gut microbiota, luminal environment, impaired barrier function and low-grade immune activation have also been reported in IBS patients.

It is estimated that about 30% of the UC patients and 40% of the CD patients in remission experience symptoms that resemble IBS (IBS-like

symptoms),[54,90-93] which makes differentiation between an upcoming flare and functional symptoms a great challenge, particularly for patients experiencing diarrhea. Shared pathophysiological mechanisms in IBS and IBD have been proposed to explain IBS-like symptoms. For example, impaired barrier function leading to increased mucosal permeability,[94-96] imbalanced gut microbiota[97,98] and increased circulation of pro-inflammatory cytokines,[99,100] have been described in both conditions. However, it is unclear if IBD patients experience IBS-like symptoms while in remission, or if these symptoms in IBD are a result of unresolved subclinical inflammation. Thus, whether IBD and IBS may be considered part of the same disease spectrum needs to be clarified.

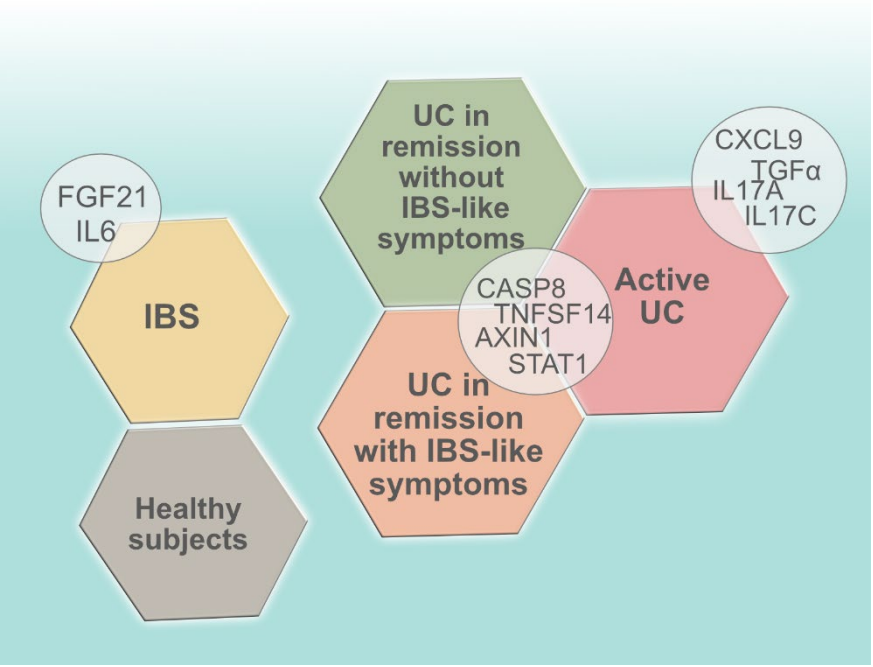


Figure 5. Graphical representation of the findings in paper I. The most important measured proteins are highlighted in the circles placed in association with the patient group(s) that they characterize best.

Understanding of the mechanistic differences between IBD and IBS, and if their immunopathogenesis might overlap, is important to improve diagnosis and patient care. Focus given to investigating IBS-like symptoms in IBD in relation to IBD and to IBS is therefore crucial to differentiate the different phenotypes of disease. In a study by our

group, investigating systemic inflammatory protein profiles in UC, IBS and healthy subjects (Paper I),[101] we observed that serum levels of fibroblast growth factor 21 (FGF21) and the pro-inflammatory cytokine IL-6 were increased in IBS patients when compared to healthy subjects, suggesting that inflammatory mechanisms are involved in IBS (Figure 5). Although an elevated FGF21 has not been described previously in IBS, an increased IL6 is in line with earlier findings identifying elevated inflammatory markers in IBS,[99,102,103] often used as basis to argue that IBS and IBD could be driven by similar mechanisms. FGF21 is an atypical growth factor that functions as a hormone regulating fatty acid metabolism. However, in our study we also demonstrated higher circulating levels of TNF super family member 14 (TNFSF14), axin 1 (AXIN1), caspase (CASP)8 and signal transducer and activator of transcription 1 (STAT1) in UC patients in remission with IBS-like symptoms when compared to IBS patients, suggesting that different mechanisms drive these entities (Paper I) (Figure 5).[101 104]

When comparing patients with UC in remission with and without IBS-like symptoms, no evidence supported differences in the systemic inflammatory protein profiles between the two groups (Paper I). Our finding contrasts with a previous study reporting epithelial barrier dysfunction in IBS patients and IBD patients in remission with IBS-like symptoms as compared to IBD patients without IBS-like symptoms and healthy subjects.[105] It is possible that the low-grade inflammation hypothesized to cause IBS-like symptoms is limited to the mucosa, and may not be detectable systemically.

3 IMMUNOPATHOGENESIS OF IBD

The mechanisms behind the immunopathogenesis of IBD are yet to be fully unveiled. Still, a dysregulated mucosal immune response, characterized by an overactive innate immune system along with activated effector T cells, an excessive presence of plasmocytes with increased antibody production, as well as an elevated secretion of pro-inflammatory mediators are considered the central drivers of IBD (Figure 6).[47] In the next sections, the different components involved in IBD immunopathogenesis will be discussed in detail.

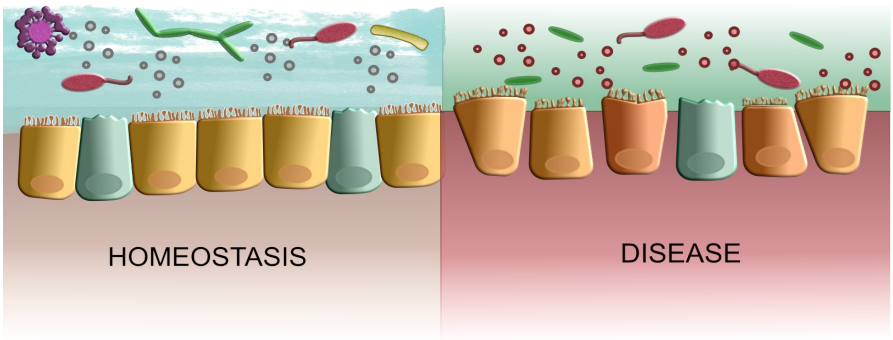


Figure 6. Schematic representation of the epithelial barrier environment in health (homeostasis) and disease. In homeostasis (left), the gut microbiota is diverse and sustains a healthy secretion of SCFAs, which contributes to epithelial health. In disease, (right) a decreased microbial diversity and altered metabolite composition negatively affect epithelial barrier integrity and favor pro-inflammatory responses.

3.1 INNATE IMMUNITY AND ITS ROLE IN IBD

The mucus layer and the intestinal epithelial barrier separating the luminal environment from the lamina propria compose the first line of defense in the innate immune system. Several studies, including genome wide studies and experimental models suggest that epithelial impairment may be an early event leading to IBD.[106-109] Abnormal secretion of mucus leading to increased permeability for luminal contents has been described in both UC and CD.[110] Further, altered expression of AMPs, and junctional proteins, such as E-cadherin and claudins, were observed in the intestinal mucosa of IBD patients in both active and quiescent disease.[105,111-114] However, it is still unclear whether

epithelial barrier disruption is a primary defect or a consequence of inflammatory response.

A number of innate immune cells are involved in IBD pathogenesis. Following barrier disruption, secretion of chemokines, including CXCL1, CXCL5 and CXCL8, leads to rapid and robust recruitment of neutrophils to the lamina propria,[115] which increase reactive oxygen species (ROS), and production of several pro-inflammatory and antibacterial molecules, such as calprotectin, lactoferrin, lysozyme, defensins and metalloproteinases (Figure 7).[116] Subsequent translocation of luminal content is sensed by PRRs expressed by APCs, fibroblasts and enterocytes, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which increases the production of pro-inflammatory cytokines, such as TNF, IL6, IL18 and IL1β (Figure 7).[117,118] An imbalance between tolerogenic signals through TGFβ, TSLP and RA, and pro-inflammatory signals, has been described in IBD, conditioning DCs into an inflammatory state (Figure 7).[119,120]

Among cytokines secreted by innate immune cells in IBD, major focus has been given to TNF. In IBD, large amounts of both membrane-bound and soluble TNF are produced by MΦ, DCs and stromal cells,[121-123] resulting in local and systemic pro-inflammatory effects that amplify and sustain intestinal chronic inflammation. TNF has been shown to promote disruption of the epithelial barrier by inducing necroptosis of IECs through activation of CASP8, and causing tight junction dysregulation.[124,125] When it comes to other immune cells, TNF activates MΦ and effector T cells, and sustain transcription of pro-inflammatory cytokines.[117] Fibroblasts also amplify TNF signaling through membrane-bound TNF, resulting in upregulation of oncostatin-M (OSM) receptor, production of matrix metalloproteinases (MMP) and secretion of chemokines and cytokines such as CXCL9, CCL2, IL6 and TNFSF14 (Figure 7).[126,127] Monoclonal antibodies (mAbs) targeting membrane-bound and soluble TNF, such as infliximab, adalimumab, golimumab and certolizumab pegol were the first biologic drugs to successfully induce remission in IBD patients. Apart from neutralizing soluble TNF from exerting its inflammatory effects, anti-TNF mAbs are believed to induce apoptosis of TNF secreting cells by interrupting the positive feedback loop and/or reverse signaling through membrane bound TNF.[128]

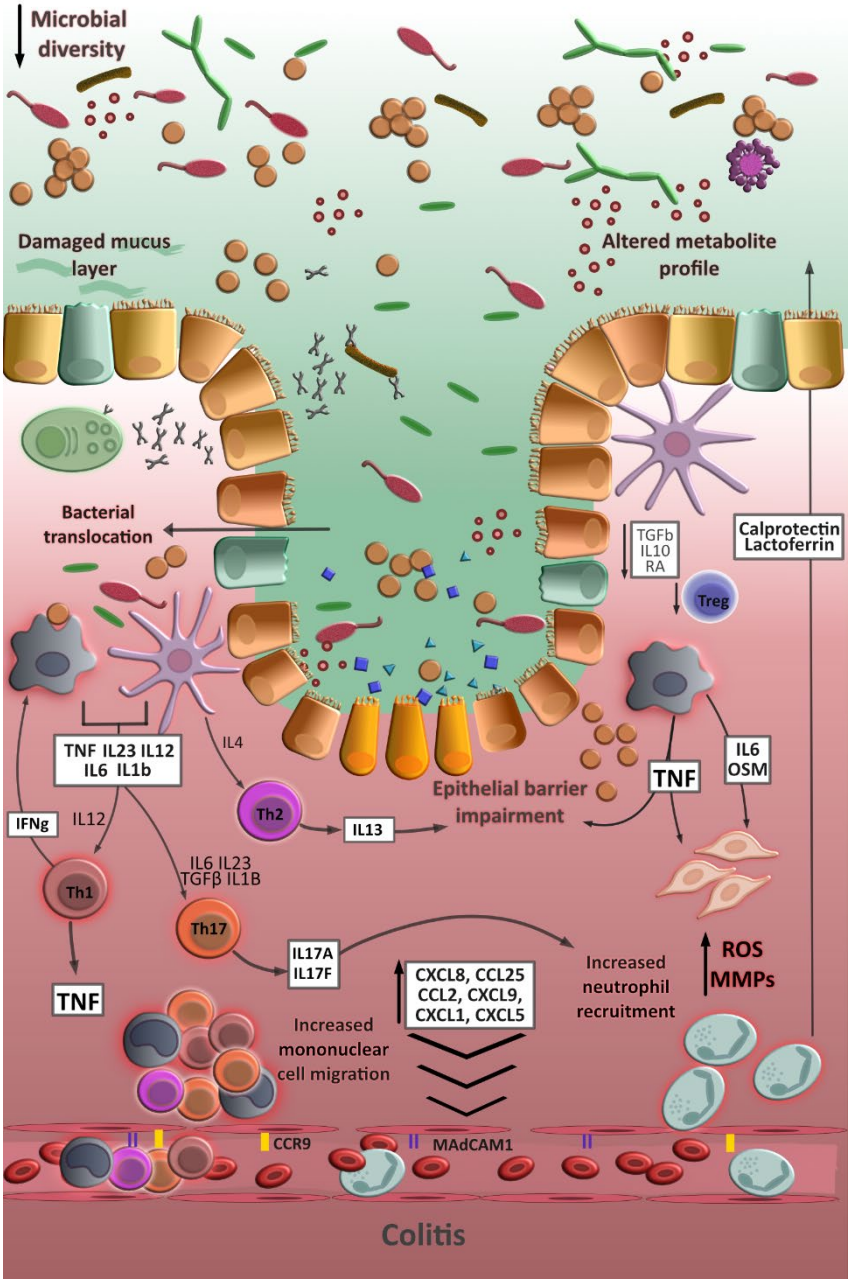


Figure 7. Representation of the main cell, chemokine and cytokine interactions in the inflamed mucosa in IBD.

In addition to TNF, excessive secretion of other cytokines involved in innate immune responses, including IL6, IL1β and IL18 have been shown to be increased in IBD.[129] IL6 exerts pro-inflammatory effects in multiple cell targets by inducing chemokine mediated cell trafficking, differentiation of CD4+ Th cells and preventing apoptosis.[130,131] Moreover, inflammasome activity driven by microbial sensing induces cleavage of pro-IL1β and pro-IL18 into IL1β and IL18, which results in recruitment of monocytes, angiogenesis and amplification of the pro-inflammatory response.[132] Particularly for IL1β, exacerbation of the mucosal inflammation may be explained by its participation in boosting Th17 cell activity.[133]

A separate family of innate cells has recently gained interest in IBD immunopathogenesis. Innate lymphoid cells (ILCs) play an important role in maintaining gut homeostasis and act as a bridge between the innate and adaptive immune systems. Due to their similarity with CD4+Th cells, they have been divided in three subtypes, ILC1, ILC2 and ILC3 based on the expression of Th1, Th2 and Th17-cytokines respectively.[134] Evidence indicates a strong presence of ILCs in the mucosa of IBD patients, particularly of ILC1 and ILC3.[135] Their activation can occur via direct microbe sensing by certain PRRs, such as TLR2[136] and/or cytokine signaling;[137] more specifically IL12 and IL18 for ILC1 polarization, and IL23 for ILC3.[138] Large amounts of ILC-derived IL17A, IL22, IFNγ and TNF has been observed in the mucosa of IBD patients,[139] which may be of relevance for disease pathogenesis.

The importance of the innate immune system to the pathogenesis of IBD is further highlighted by the number of genetic variants associated with susceptibility to IBD, discussed later in this thesis. In our study investigating systemic inflammatory protein profiles (Paper I), increased serum levels of axin-1 (AXIN1), sulfotransferase 1A1 (St1A1), TNFSF14 and CASP8 were consistently increased in UC patients irrespective of disease activity when compared to non-UC subjects (Figure 5). Out of these proteins, elevation of TNFSF14 and CASP8 levels is a result of TNF activity linked to innate immune response. Moreover, patients with active UC displayed higher levels of CXCL9 (Paper I), in agreement with previous research describing elevated circulating CXCL9 in IBD patients and experimental colitis models when compared to healthy controls.[140,141] The signature was further validated in a separate cohort of UC patients with active disease, UC patients in remission and IBS patients, effectively differentiating UC patients from IBS (Paper I) (Figure 5).

3.2 ADAPTIVE IMMUNITY AND ITS ROLE IN IBD

For many years, CD was considered a Th1 mediated disease whereas UC was thought of as an atypical Th2 driven disease. This paradigm was supported by the imbalance observed in the mucosal expression of Th1/Th2 cytokines. A more pronounced Th1 response, with increased levels of IFNγ, has been seen in CD when compared to UC.[142,143] Elevated levels of IFNγ activate other immune cells from the innate compartment, such as MΦ and NK cells, which in turn amplify the immune response by sustaining TNF secretion.[117] In contrast, higher levels of Th2 cytokines, such as IL13 and IL5, have been observed in the UC mucosa. IL13 is believed to participate in the immunopathogenesis of the disease by disrupting the epithelial barrier, which contributes to the translocation of luminal content to the lamina propria and activation of local immune responses (Figure 7).[144] The Th1 and Th2 cell lineages exert further cross-regulatory effects on one another through cytokine signaling. IFNγ produced by Th1 cells suppresses the proliferation of Th2 cells, which in turn antagonizes Th1 cell proliferation by secreting IL4.[145] However, inconsistencies in this theory, such as low levels of IL4 expressed in UC, shows this is likely an oversimplification of a more complex immunopathogenesis.[142] Further, directed therapies towards Th1 or Th2 effector cytokines, IFNγ and IL13 respectively, have not been successful in inducing remission in IBD patients,[146,147] suggesting that mechanisms independent from Th1/Th2 responses also sustain chronic inflammation.

Discovery of other T-cell populations, such as Th9 and Th17, and innate lymphoid cells (ILCs) and their role in IBD has challenged the classical Th1/Th2 paradigm.[148,149] The Th17 subset is generated in the presence of IL6, TGFβ and IL23, and secretes large amounts of IL17A, which induces epithelial secretion of CXCL8, with subsequent recruitment and activation of neutrophils (Figure 7).[150] Elevated serum levels of IL17A and IL17C was observed in UC patients with moderate to severe disease activity when compared to UC patients in remission and healthy subjects (Paper I), in agreement with previous literature showing that IL17A is a key cytokine in UC. Furthermore, increased levels of AXIN1 in patients with UC in active disease or in remission (Paper I) supports the importance of the Th17 subset in mucosal inflammation. AXIN1 has been described to participate in the phosphorylation of transcription factor Smad3 that regulates differentiation of Th17 cells.[151] However,

treatment targeting IL17A with neutralizing mAbs has been shown to worsen colitis,[152] suggesting a dual role for IL17A. In contrast, drugs targeting the differentiation of Th17 cells, such as anti-IL12/IL23 p40 subunit ustekinumab, have been shown to be effective for both UC and CD.[153-155] As the p40 subunit is shared by IL12 and IL23 receptors, it is possible that the simultaneous interference on Th1 and Th17 differentiation provides better therapeutic results than targeting single effector cytokines secreted by adaptive immune cells.

3.3 ENVIRONMENTAL FACTORS AND EPIDEMIOLOGY

Modern industrial development in the 20th century was followed by profound changes in human lifestyle and concomitant increase in complex chronic inflammatory illnesses, such as IBD.[42,156] Various components of the modern life, such as antibiotics, diet and smoking, are currently known to modify the intestinal immune response and affect the microbial composition. For example, observational studies report a particularly increased risk for developing IBD in adults who have suffered an episode of gastroenteritis and children frequently exposed to antibiotics.[43,157] Such events are likely associated with an imbalanced microbiota and the breach of the epithelial barrier by commensal bacteria.

Marked development in medical treatments, vaccination, infection control and urbanization have also been correlated with the increased prevalence of chronic inflammatory diseases and allergy worldwide.[158,159] These observations form the basis for the "hygiene hypothesis" which suggests that decreased exposure to microorganisms during childhood contributes to the failure of proper immune maturation and the later development of chronic inflammatory disorders.[160] Indeed, probiotic bacteria have been demonstrated to induce a tolerogenic phenotype in dendritic cells *in vitro*,[161,162] suggesting that a healthy gut microbiota is important for gut homeostasis.

3.4 GENETICS

Genetic heritage has long been considered relevant in IBD pathogenesis, but it was not until the first population-based studies with twins and family cohorts that the relative contribution of genetic

and environmental factors was established.[163-168] For monozygotic twins, studies report a 20-50% concordance rate for CD and a 16-19% concordance rate for UC. However, dizygotic twins brought up in the same household show concordance rates below 10% for both CD and UC.[169] When it comes to family studies, the rate at which patients with IBD report a family case ranges from 8-14%.[167] Still, genetics is most often not the sole explanation to why certain individuals develop IBD as it is estimated that genetics only explain 20-25% of the cases.[46]

Technological advances in genome sequencing opened a door for large genome-wide association studies (GWAS) in IBD, which until now have identified approximately 200 genetic variants linked with IBD.[46] Among them, the most well-studied associations with disease pathogenesis are variants related to PRR nucleotide-binding oligomerization domain protein-2 (*NOD2*), caspase recruitment domain-containing protein 9 (*CARD9*); autophagy related genes immunity-related GTPase family M (*IRGM*) and autophagy related 16 like 1 (*ATG16L1*); cytokines IL23 and IL10, and IL10 receptor subunit α (*IL10RA*), the last two associated with early-onset IBD.[170,171] Pathway analyses show that *NOD2* and *ATG16L1* are closely connected and activation of *NOD2* by pattern recognition may activate autophagosome formation via *ATG16L1* for degradation of bacteria during steady state.[172] Further, IL23 expression is involved in the differentiation of Th17 cells towards an anti-inflammatory phenotype in addition to stimulating mucosal secretion of IL22 that sustains epithelial AMP production.[173] Malfunction of any of these processes, which are important for sustaining epithelial barrier integrity and tolerance, may result in chronic inflammation. However, the mere presence of these genetic variants in a given individual may not be sufficient to trigger disease on its own.

3.4.1 DYSFUNCTIONAL MUCOSAL GENE EXPRESSION

Investigating possible ways by which the environment and the luminal content influence whether a gene is silenced or active, and contribute to an IBD prone phenotype seem like a logical path forward. Since epigenetic modifications are heritable but dynamic, meaning that they are influenced by the exposure to different stimuli, they may explain why contextual changes in diet, habits, medication and environment over generations are resulting in an increased IBD incidence worldwide. Several studies have demonstrated an association between epigenetic mechanisms and certain clinical features, such as disease severity and steroid dependency for UC,[174,175] but this is still an emerging field.

Not all patients carry the genetic variants known to be associated with IBD. However, the genetic vulnerabilities revealed by IBD GWAS studies have uncovered possible network of mechanisms contributing to its immunopathogenesis. Further, the identification of key processes contributing to disease has been vital for the development of new biologic therapies, such as ustekinumab, modulating the IL12/IL23 signaling pathway. With the development of analytical methods investigating gene function, such as gene expression and pathway enrichment analyses, the understanding of the active processes involved in the immunopathogenesis of IBD has improved.

In paper II, we have explored PCR (polymerase chain reaction) methods assessing messenger RNA (mRNA) to analyze gene expression in colon samples from IBD patients with active disease and in remission. In this study, a multiplex panel was chosen, including almost 800 genes involved in the host-response, for a targeted analysis (Nanostring nCounter Host-Response panel, Nanostring technologies). The relatively broad panel, in comparison to the more limited array chosen in Paper III, allowed us to identify patterns among the expressed genes that suggest positive or negative regulation of their corresponding pathways.

When comparing the mucosal gene expression in healthy subjects to patients with IBD, we found sustained downregulation of IL17 signaling, alkaline phosphatase kinase 1 (ALPK1) and autophagy pathways in IBD patients, independent of disease activity (Paper II). This is in agreement with the theory that the dysfunctional immune response in IBD is associated with microbial sensing, cytokine signaling and autophagy defects, supported by the gene variants commonly linked to IBD risk (Figure 8).

NOD2 polymorphisms, associated mainly with CD, have been regarded as a loss of function mutation, leading to a weaker binding to bacterial peptidoglygans.[176] In turn, secretion of α-defensins and activation of autophagy pathways are compromised. Indeed, the autophagy pathway was downregulated in active IBD and quiescent UC when compared to healthy individuals in our study (Paper II). However, mucosal NOD2 expression was increased (Figure 8). Considering that only a minority of the patients included in the study have CD, it is possible that in UC, dysregulation of the autophagy pathway is independent of NOD2. Further, downregulation of the lysosome pathway in patients with quiescent UC (Paper II) may contribute to dysregulation of autophagy,

as lysosomes are essential for breaking down intracellular waste products.

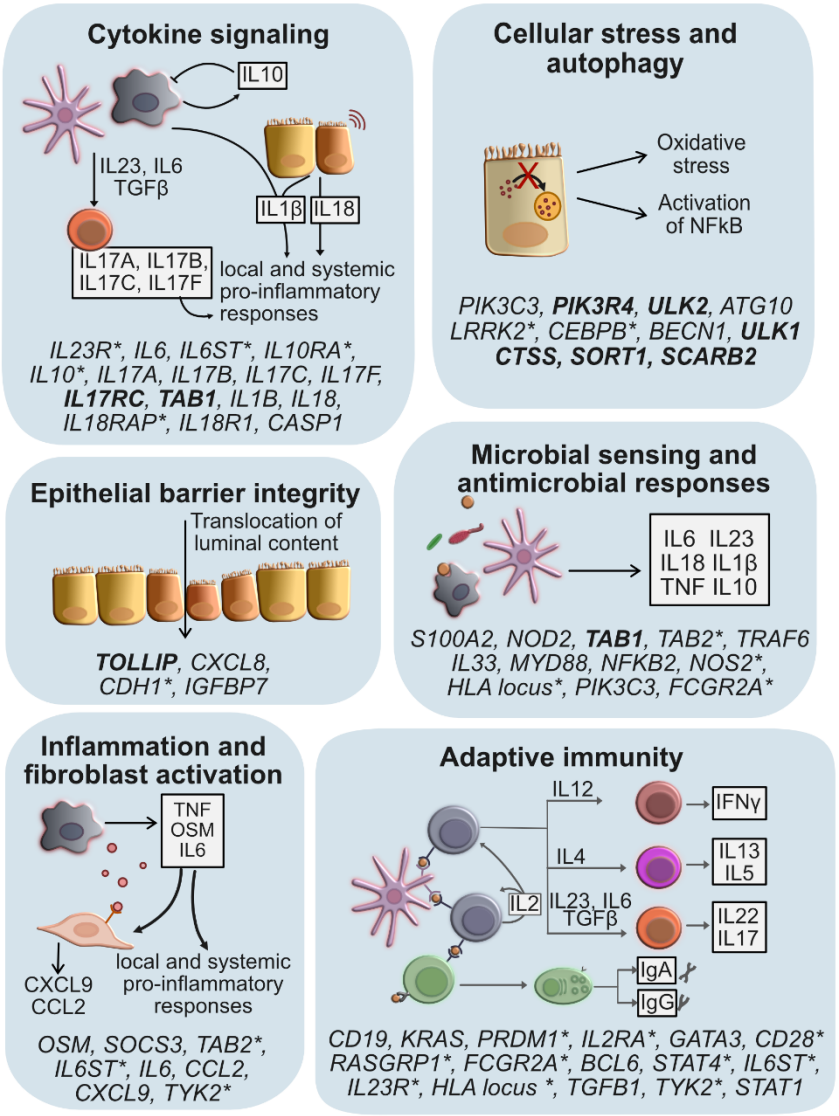


*Figure 8. Graphical summary of the findings in paper II. Each box represents a group of affected pathways identified in the study. Genes associated with a certain group of pathways and found to be increased in gene expression in our study are listed in the bottom of the box. Genes listed in bold were persistently altered in active disease and in remission. *Genes whose polymorphisms are described in the literature as associated with IBD.*

Autophagy is an essential innate immunity response found in all cell types, which mediates removal of intracellular waste and phagocytosed content for cell renewal.[177] It can be activated via NOD2 signaling but also cytokine signaling, intracellular signals and PRRs can induce the process.[178] For example, the vitamin D receptor activates the autophagy cascade,[179] but has been shown to be less expressed in UC patients and genetic variants are associated with the disease.[180] Further, dysfunctional activation of protein kinase mTOR via PRR, inhibiting the autophagy cascade, has been reported in UC patients.[181] Autophagy negatively controls inflammasome activity by promoting degradation of assembled inflammasomes and downregulating IL1β expression.[182,183] Therefore, impaired autophagy is associated to increased IL1β and IL18 during inflammation. Sustained downregulation of the autophagy pathway could explain our finding of increased gene expression of IL1β and IL18 in UC patients during disease activity (Paper II).

When it comes to IL17 signaling, the mucosal expression of the IL17 family of pro-inflammatory cytokines was increased in patients with active IBD when compared to healthy subjects (Figure 8) (Paper II). However, when analyzing the overall regulation of the pathway, IL-17 signaling, including expression of *IL17RC*, were consistently lower in active disease and remission as compared to healthy subjects (Paper II). Interestingly, disruption in autophagic activity has been reported to enable the release of IL-23 and IL-1 cytokines, consequently favouring Th17 differentiation and secretion of IL17.[118,184] However, we did not observe a dysregulated Th17 differentiation pathway in our patient cohort (Paper II). Effective signal transduction for IL17A and IL17F depends on the upregulation of a heterodimeric receptor complex consisting of the ubiquitously expressed IL17RA and the inducible receptor subunit IL17RC.[185] The absence of either receptor subunits leads to ineffective signaling.[186] Thus, our data suggests a persistent impairment on the mucosal IL17A/F signaling in IBD, which may affect host response and clearance of extracellular pathogens.[187] Indeed, evidence demonstrating that anti-IL17 treatment triggers onset or exacerbation of IBD, further supports that the IL17 signaling pathway is essential for gut homeostasis and prevents hyperactive innate inflammatory processes.[188]

3.4.2 CROHN'S COLITIS: THE INTERSECTION BETWEEN CD AND UC?

As UC and CD affect the GI tract differently, it has for long been concluded that they are driven by distinct mechanisms. However, the classical description of CD as a Th1 and UC as a Th2 mediated disease is likely an oversimplification of a more complicated inflammatory response. Recently, studies investigating the mucosal, serological and microbial characteristics of ileal and colonic CD have shown that they are more likely distinct entities.[189,190] Yet, it is still not established whether Crohn's colitis is more similar to UC than other forms of CD. While some studies report immunological differences between them, others do not find major discrepancies, which challenges the current diagnosis definition.

Indeed, our study (Paper II) investigating gene expression in biopsies collected from active sites of colitis (CD and UC) demonstrated similar mucosal transcriptional profiles in both entities. The study included a relatively small number of CD patients, with mixed disease phenotypes (L2 and L3), which limits the generalizability of our findings. Ideally, a larger cohort of CD patients, grouped according to their disease location, should be studied and compared to UC and CD ileitis for better characterization. Still, our data suggest that UC and colonic CD have similar mucosal immune profiles although the colonic macroscopic presentation differs.

3.5 LUMINAL ENVIRONMENT IN IBD

Whether imbalance of the intestinal microbiota composition is the initial trigger or the result of inflammation is unclear. Epithelial barrier defects, modified expression of AMPs, decreased microbial diversity and altered fecal metabolite profiles, have all been reported in patients with IBD supporting the critical role of a dysfunctional intestinal barrier for disease development.[106,107] However, the mechanisms behind immune dysfunction and the host-microbial crosstalk are yet to be fully understood.

The composition and diversity of the gut microbiota are crucial factors for the development of IBD.[191] Higher instability in the microbiota composition in CD patients when compared to UC has been described.[192,193] A longitudinal study including UC patients has showed that their microbiota composition is highly stable over time and does

not vary significantly with disease activity fluctuations,[192] which supports the idea that microbial imbalance maybe be present before disease onset. Further evidence suggests that a decreased abundance of *Bifidobacterium longum, Eubacterium rectale, Faecalibacterium prausnitzii,* and *Roseburia intestinalis,* and increased abundance of *Escherichia coli* and *Ruminococcus spp.*, contributes to the immunopathogensis of both UC and CD.[194] Low abundance of *Roseburia* is significantly associated with expression of the IBD-related genes *NOD2, ATG16L1* and *IRGM*,[195] suggesting that the crosstalk between the mucosa and *Roseburia* involves the autophagy pathway. Moreover, loss of butyrate-producing bacteria, such as *F. prausnitzii*, may affect epithelial metabolism, since butyrate serves as an important source of energy for enterocytes.[196] However, the specific role of individual microorganisms on the pathophysiology of IBD remains to be determined.

Another fundamental question regarding microbiota and the pathogenesis of IBD is whether microbial imbalance alone is sufficient, or if a combination with environmental and immune alterations are required to trigger disease. Studies comparing dextran sulfate sodium (DSS) induced colitis in conventional and germ-free mice have shown that the microbiota is essential for colitis development.[197] However, critical hallmarks in human development shaping the gut microbiome, such as delivery mode, do not seem to affect the risk of developing IBD[198,199], which suggests that a combination of variables are most likely necessary for immunopathogenesis of IBD.

If the gut microbiota composition is highly stable over time, and individual fluctuations do not seem to correlate with disease activity, it is possible that shifts in the microbial metabolism play a more important role in triggering flares. Metabolites are a dynamic imprint of the present luminal environment, habits of the host and a translation of the microbial activity rather than diversity fluctuations as seen in studies focusing on the microbiota composition.[200] Indeed, previous studies have reported alterations in stool metabolite compositions in IBD, such as decreased SCFAs levels and overall decreased metabolite diversity.[201,202] In agreement with previous research, showing that fecal metabolites may be used as non-invasive biomarkers determining disease activity,[201,203,204] untargeted metabolite analysis in our small cohort of UC patients in disease flare and in remission, revealed a unique fecal metabolite composition pattern for each group (Paper III). These combined findings suggest that microbial activity rather than

diversity is important for disease pathogenesis. Gut metabolite composition may be an interesting non-invasive tool for disease assessment and diagnosis in the future.

3.6 COLITIS ASSOCIATED COLON CANCER

For paper III, investigating the effects of luminal factors on *in vitro* systems, we included a small cohort of patients with colon cancer to compare with IBD and IBS. Colon carcinoma is a the third most common cancer worldwide, and an important complication in IBD.[205] Colon cancer that develops in patients with IBD colitis is named colitis-associated cancer (CAC) and is a major cause of death among IBD patients.[206] The risk of CAC starts to increase after 8-10 years from disease onset and continues to increase over time, which suggests that disease duration is an important risk factor.[207] Other variables that have been shown to contribute to cancer risk are younger age at disease onset, family history of colorectal cancer in first-degree relatives and concomitant occurrence of primary sclerosing cholangitis.[52,207]

Overall, IBD patients are 2-6 times more likely to develop colon cancer than the general population, and its manifestation takes place at a younger age than sporadic colorectal cancer. Geographical heterogeneity in relation to risk rates have been described, which may reflect environmental differences but also variations in clinical management when it comes to surveillance and chemoprophylaxis.[52,208,209] For example, Scandinavian countries have reported lower risk rates for CAC in both UC and CD when compared to similar cohorts in the United States and United Kingdom.[52,210] Nevertheless, certain biases in the assessment of risk of CAC, such as aging population and heterogeneity of disease phenotypes, may affect the interpretation of these results.

Although chronic inflammation and epithelial damage occurring in IBD are known factors linked to tumorigenesis,[211] CAC does not appear to follow the adenoma-carcinoma sequence as seen in sporadic colon cancer. Instead, evidence supports that CAC stems directly from tissue dysplasia following chronic inflammation.[212] Sustained activation of transcription factor NFκB via TNF signalling and TLR activation seems to drive the chromosomal and microsatellite instability associated with early dysplasia in IBD.[213] As a result, increased expression of key genes such as tumour suppressing gene *TP53*, cell cycle regulatory gene *BCL2,* stress response genes *PTGS2* and *NOS2*, and pro-inflammatory

cytokines *TNF* and *IL12* are involved in carcinogenesis. Moreover, animal studies suggest that defective signalling of tumour suppressor gene p53 could be an early event leading to dysplasia,[214] in difference from sporadic colon cancer, where p53 mutations are late events.[215] In contrast, mutation in the adenomatous polyposis coli (*APC*) oncogene, involved in early adenoma formation in sporadic colon carcinoma,[216] represents a late transformation in CAC.[217]

In parallel with a dysfunctional immune response, altered gut microbiota signatures have been described in different types of colon cancer, including CAC. In contrast to sporadic CRC where studies consistently points towards an increased abundance of *Fusobacterium* spp. and *Bacteroides fragilis*,[218-221] increased abundance of *Enterococcus* spp. and *Streptococcus* genus has been observed in CAC.[222] In both cases, a surge of pro-carcinogenic microbial metabolites derived from the metabolism of bile acids, choline, carnitine, sulfur and nitrogen compounds may be involved in modulation of the mucosal immune responses.[223] Since chronic intestinal inflammation and epithelial damage are known to affect microbial composition,[211,224] it is possible that the prolonged inflammation associated with IBD supports a pro-carcinogenic metabolism in the gut microbiota, which in turn feeds dysplasia.

4 *IN VITRO* MODELS FOR STUDYING INTESTINAL DISEASES

Numerous studies attribute the rising prevalence of IBD and other intestinal diseases to environmental and cultural changes affecting the gut microbiome, epigenetics and the mucosal immune system responses.[225-229] This is further supported by the reduced microbial diversity observed in IBD patients when compared to healthy individuals.[98,230] Current strategies to investigate the relationship between the local microenvironment, immune response and barrier integrity have focused on characteristics of the immune response and microbiota composition separately, due to the complexity of integrating systems. Thus, a major challenge for experimental approaches when studying the gut-barrier crosstalk is the development of models that integrate the luminal environment and epithelium, particularly in the context of disease.

Experimental models to study intestinal diseases have mainly been focused on animal studies, particularly studies in mice. The most common approaches are chemically induced colitis models, such as DSS (UC)[231] and trinitrobenzene sulfonic acid models (UC and CD).[232] However, genetically engineered models, such as IL10 or TGFβ knock-out mice, have been widely used to understand the involvement of specific genes in disease.[233,234] Although animal models have made important contributions to the field, none of them fully replicate the multiple factors related to IBD pathogenesis.[235] For instance, chemically induced colitis models are often self-limiting, which does not reflect the chronic character of IBD. Moreover, animal models may fail to consider the variability of environmental stimuli involved in disease pathogenesis.

Complementary to animal studies, *in vitro* techniques using cell lines and human stem cell-derived organoids may provide additional insight into gut physiology, host-microbe and cell-cell interactions.[236] Functional and structural characteristics of the epithelial interface under controlled conditions has been widely studied with the help of immortalized cell lines, such as colon carcinoma derived Caco-2, T84 and HT29 cells. However, immortalized cell lines only represent a fraction of the diverse IEC types in the intestinal epithelium and thus constitutes a simplistic model to study gut barrier interactions. In contrast, human-derived organoid systems, generated from intestinal

stem cells, differentiate into the various IEC types, which makes it a more representative alternative of the physiological gut barrier.[237]

Strategies to simulate the influence of microbial metabolites or dietary products on the epithelial barrier have focused on controlled experiments testing one stimulus at a time, which does not reflect the gut luminal environment, where a complex cocktail of metabolites may have an effect on the epithelium. Evidence described by us (Paper III) and others[238-240] report that the fecal metabolite composition differs between intestinal diseases, in this case IBS, UC and colon cancer. Taken together, data indicate that fecal samples could be used as a proxy for the luminal microenvironment of its donor. Indeed, filtered ultra-centrifuged fecal supernatants from IBS patients and healthy subjects have been used in a study by our group evaluating their effects *in vitro* on human colon-derived organoids (colonoids).[241] In this study, colonoids cultured with fecal supernatants from IBS patients and healthy subjects displayed distinct gene expression profiles suggesting that the luminal content in disease states is possibly involved in epithelial alterations that might lead to symptoms.

A similar setup was tested by us including two different *in vitro* cell culture models stimulated by fecal supernatants from IBS, UC and colon cancer. In our study (Paper III), we exposed Caco-2 cell monolayers and three dimensional apical-out colonoids from a single donor to patient-derived fecal supernatants to determine the effects of the luminal content on the gene expression patterns of the intestinal epithelium. As a result, distinct gene expression patters of the two cell culture models stimulated with fecal supernatants from different patients groups were recorded (Figure 9). Discussion of the culture models and results are described in the following sections.

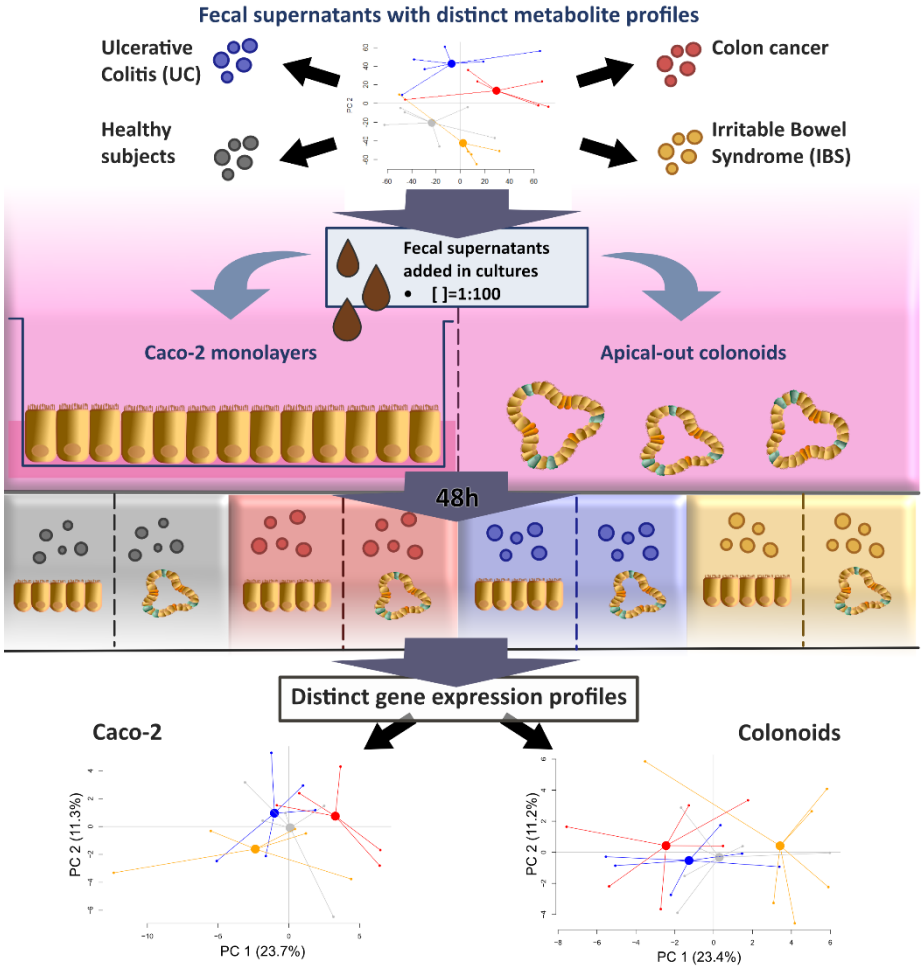


Figure 9. Graphical representation of Paper III. Fecal metabolite composition was analyzed for patients with UC, IBS, colon cancer and healthy subjects. Caco-2 monolayers and colonoids derived from a healthy donor were stimulated with fecal supernatants from the study groups for 48h and gene expression analysis was performed, which demonstrated distinct gene expression profiles.

4.1 COLON CARCINOMA DERIVED CACO-2 CELLS

Colon carcinoma derived Caco-2 cells have been used since the 1980's for studying the intestinal epithelial barrier. *In vitro* models exploring the effects of stimulating Caco-2 cells with specific microbial metabolites and food nutrients are relatively common, and have demonstrated clear modulatory effects of the microbiota on colonocytes.[242-244]

Relatively simple to culture, Caco-2 cells can be further differentiated into colonocytes-like cells and polarized using Transwells® culturing techniques, which facilitates separate access to apical and basolateral compartments for *in vitro* stimulation (Paper III).[245] Although being of cancerous origin, when cultured for 15 days or longer they shift their gene expression into a colonocyte-like phenotype[245] making them a proxy for the colonic epithelium *in vitro*. In our study, Caco-2 cells were seeded in Transwell® inserts and cultured for 20 days before stimulation with fecal supernatants (Paper III). The time between seeding and differentiation was optimized in a pilot study, following alkaline phosphatase (ALP) secretion, a colonocyte differentiation marker[246], through a colorimetric assay. Increased and stable secretion of ALP is a sign of colonocyte differentiation.[246]

Instead of stimulating Caco-2 cells with single metabolites or nutrients, we used fecal supernatants to understand the effects of the luminal microenvironment on the colonocytes. Interestingly, increased cell viability was observed in Caco-2 monolayers stimulated with fecal supernatants from healthy subjects when compared to untreated controls (Paper III). Possibly, fecal metabolites present in the supernatants are consumed by the colonocytes for energy production, which improves cell viability.[247] Further, SCFAs have been described to have a positive effect on gut barrier maintenance, participating in pH control modulating immune responses.[242,248]

When comparing the effects of fecal supernatants from healthy subjects with the effects from patient groups, we observed that the Caco-2 monolayers assumed different gene expression patterns. Despite our small sample group, these results suggest that fecal supernatants possibly reflect the luminal microenvironment of the donor and influence the epithelial barrier behavior.

4.2 HUMAN DERIVED COLON ORGANOIDS

Human derived colon organoids is a promising technique to study the crosstalk between the epithelial barrier and external stimuli, such as microbiota and its products. Organoids are three-dimensional (3D) cell structures that can be used for *in vitro* drug or stimulations assays and as model system for diseases. Intestinal organoids are generated from tissue-resident stem cells collected from biopsies, and are differentiated into the various gastrointestinal cell types during specific culture conditions, forming a miniaturized and simplified version of an organ.[249] The use of colon organoids derived from colon carcinoma and UC patients, for replicating disease mechanisms and develop individualized therapy strategies, have evolved during recent years.[250-252] Studies show that organoid models reproduce the genetic and morphologic characteristics of the sample donor,[253-255] supporting the exploration of these systems as an *ex-vivo* platform for individualizing care. However, establishing organoid cultures is a demanding, time consuming and expensive method that limits the widespread clinical use.

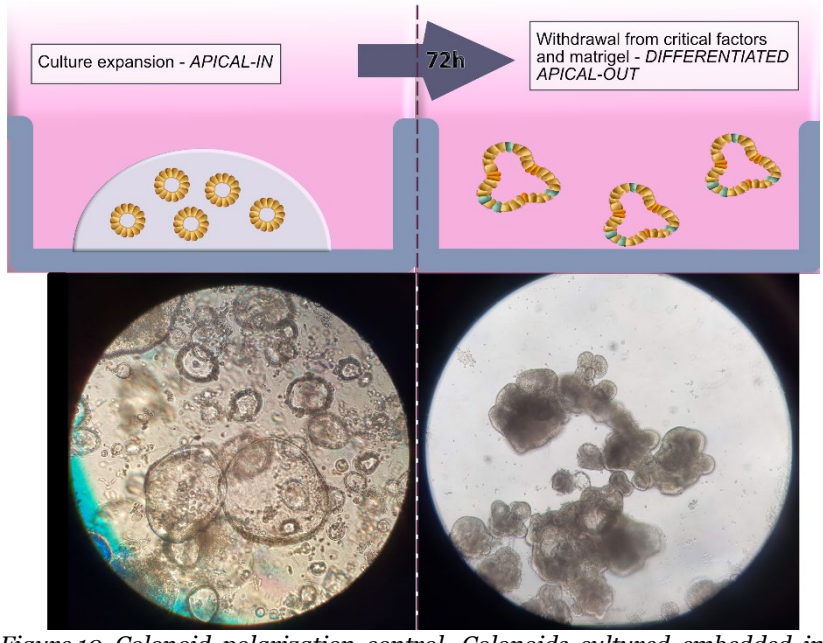


Figure 10. Colonoid polarization control. Colonoids cultured embedded in Matrigel, grow with the apical side facing inwards. Dissolution of the Matrigel and withdrawal from critical factors in the media will cause them to evert polarization and differentiate. Light microscopy (bottom row) showing apical-in colonoids to the left and apical-out colonoids to the right.

To generate colon-derived organoids, intestinal crypts were isolated from colonic biopsies collected from healthy volunteers during an endoscopic procedure. The iPSCs present in the crypts multiply and form spheric structures when cultured embedded in base membrane extract (Matrigel) simulating the extracellular matrix, and form three-dimensional "mini-intestine" spheres with the apical side facing inwards.[256]

To simulate the physiological environment, fecal supernatants must be delivered to the apical side, facing the lumen. However, assessing the apical side in apical-in colonoids can only be performed using micro-needles to inject individually each colonoid with fecal supernatants, which risks damaging the structures, wasting material and consuming an excessive amount of time to perform stimulations. To facilitate access to the apical side, earlier studies have used colonoid monolayers, where the 3D structures are disrupted, to be cultured in Transwells®,[241] similarly to the Caco-2 cells. Alternatively, colonoids may be everted, by dissolving the Matrigel, and culturing them in suspension in differentiation media for 72h. The sudden absence from the Matrigel support will lead them to evert polarization, exposing the apical side outwards (apical-out).[249] Further withdrawal from critical growth factors present in the media, will lead to differentiation from the colonoids into different cell types in of the intestinal epithelium (epithelial cells, goblet cells and eventual Tuft cells), and mimic physiological intestinal function (Figure 10).

4.3 THE LUMINAL CONTENT AND ITS EFFECTS ON THE GUT BARRIER

Studies investigating the effects of the microbiota and their metabolites on human derived intestinal organoids are rare,[241,257,258] and hitherto no study has simultaneously tested fecal supernatants from patients with different GI diseases to identify disease-specific patterns. Therefore, in our study, apical-out colonoid cultures derived from a healthy individual were stimulated with fecal supernatants collected from patients with UC, IBS and colon cancer (Paper III). Differences in gene expression between colonoids and Caco-2 monolayers treated with fecal supernatants were observed in our study (Paper III), which may be explained by the different cell types that compose the two systems. While Caco-2 cells represent only one cell type, colonoids include diverse cell types. Thus, cellular responses to external stimuli may be

different and be affected by cell-cell interactions. Further, colonoids derived from a single individual were used, and it remains to be established if the fecal supernatants would have induced a different gene expression pattern in organoids from another donor.

Interestingly, stimulation of the Caco-2 monolayer cell culture with fecal supernatants from all patient groups increased expression of tight junction associated protein *CLDN2* when compared to healthy in our study (Paper III). Upregulation of *CLDN2* has been reported as an important response to infectious diarrhea, increasing water efflux, and has been proposed as a mechanism for diarrhea predominant IBS.[259,260] In contrast to Caco-2 monolayers, apical-out colonoids consistently showed an increased expression of *IL12A* upon treatment with fecal supernatants from colon cancer, UC and IBS patients when compared to healthy subjects (Paper III). Indeed, IL12a has been proposed as a key mediator for initiating colitis in animal models, triggered by early epithelial barrier dysfunction and interaction with the gut microbiota.[261,262] However, poorer prognosis and low levels of circulating and mucosal IL12a have been reported for patients with colon cancer, indicating an anti-tumor effect for this cytokine.[263,264] This suggests that increase of *IL12A* may reflect *in vivo* immune activity triggered by luminal factors in disease settings.

Yet, stimulation of cell cultures comprising of Caco-2 cell monolayers and colon organoids, respectively, with fecal supernatants derived from CC, UC and IBS patients induced distinct gene expression profiles. This suggests that the fecal samples reflects the luminal microenvironment of the donor. Potentially, the experimental set up described by us can be used to further elucidate the crosstalk between the gut microbiome and the intestinal barrier, and improve our understanding of how local luminal factors influence pathogenesis of intestinal diseases.

5 FUTURE PERSPECTIVES

Understanding IBD as a multifactorial illness imposes challenges that traditional hypothesis-based science may not effectively approach, as it often reduces research to a few dimensions. The overwhelming complexity and unpredictability of the components contributing to disease requires a widened perspective, that takes into account human variability and the diverse phenotypes observed in clinical practice. The research described in this thesis has advanced the understanding of the disease immunopathogenesis on mucosal on systemic levels from a multivariate perspective, allowing for improved characterization of patients according to disease activity and phenotype.

Interestingly, our data suggested that TNF/CASP8 mediated epithelial barrier dysfunction and Th17 axis is of importance for disease pathogenesis in UC, and was persistently altered during active disease and in remission (Paper I). These findings were confirmed by further analysis of mucosal gene expression of patients with active and quiescent UC, revealing dysfunctional IL17, ALPK1 and autophagy signaling when compared to healthy individuals (Paper II). This suggests that perpetuated IL17 signaling and barrier function are vital for maintaining mucosal homeostasis and health. Sustained alterations during the state of remission might explain the relapse and remitting course observed in patients with IBD, and possibly, different target treatments or combination therapies during the remission period are a way forward for preventing flares in the future.

When it comes to the luminal microenvironment, disease specific metabolite profiles were different between patient groups and induced distinct epithelial barrier responses *in vitro*. Disease specific metabolite profiles, potentially reflecting different disease mechanisms, may be a promising target as a non-invasive biomarker for diagnosis and assessment of disease activity. Furthermore, *in vitro* culture systems were stimulated with fecal supernatants from various patient groups to determine if the luminal content induces distinct epithelial responses. Despite the limited number of samples, we demonstrated that the effects of the fecal supernatants from colon cancer, UC and IBS on Caco-2 monolayers and colonoids were different from healthy subjects. Potentially, the epithelial responses to addition of fecal supernatants from the different patient groups reflect the *in vivo* events associated with the respective disease. This experimental setup is a promising

strategy to understand specific mechanisms by which the luminal content interacts with the epithelial barrier and contribute to disease pathogenesis. Further improvements in cell culture techniques may allow even better exploration of the host-luminal crosstalk. For example, *in vitro* co-culture of lamina propria cells, such as MΦ and fibroblasts, with colonoid systems are already possible to maintain for a limited period of time. Hence, stimulation of such cultures with fecal supernatants from IBD patients may provide an improved replicate of the complexity of the gut barrier interactions during disease. The discovery of specific metabolites affecting the gut barrier homeostasis and their mode of action may also enable future therapies modulating the microbiota.

Disease heterogeneity is still a challenge for patient care. The interaction between the components of IBD immunopathogenesis is unique for each patient, posing an intimidating prospect for applying individualized care. Currently, it is not possible to predict disease course or therapy response, which complicates medical assistance. While the number of therapeutic options modulating immune response increases, in depth characterization of immune phenotypes during disease activity and in remission is crucial. Identifying key mechanisms orchestrating chronic inflammation, and biomarkers characterizing patient profiles, might enable the selection of the best-fitting treatments. Big data approaches, such as the ones used in this thesis, are useful tools for identifying molecular signatures that define specific phenotypes among IBD patients. While single markers have not yet been sufficient to predict outcomes, development of analytical methods for standardized clinical use, and validated signature panels combined with multivariate algorithms, may be a future possibility to classify IBD patients into profiles that take into account disease heterogeneity.

Altogether, future studies are warranted to understand the interplay of mechanisms involved in initiating and sustaining chronic inflammation, and explain the different phenotypes observed among patients with IBD. Comprehensive characterization of IBD patients will be essential to define phenotype-specific molecular signatures that enable individualized treatment strategies. Further improvement of *in vitro* and experimental techniques to model the luminal microenvironment are crucial for in depth understanding of the crosstalk between the luminal microenvironment and the gut barrier in patients with intestinal diseases. Potentially, these studies combined with improved or new analytical tools, will advance the understanding

of patient diversity, so we can take the next step towards an effective individualized treatment for IBD.

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