Gut mucosal gene expression in inflammatory bowel disease

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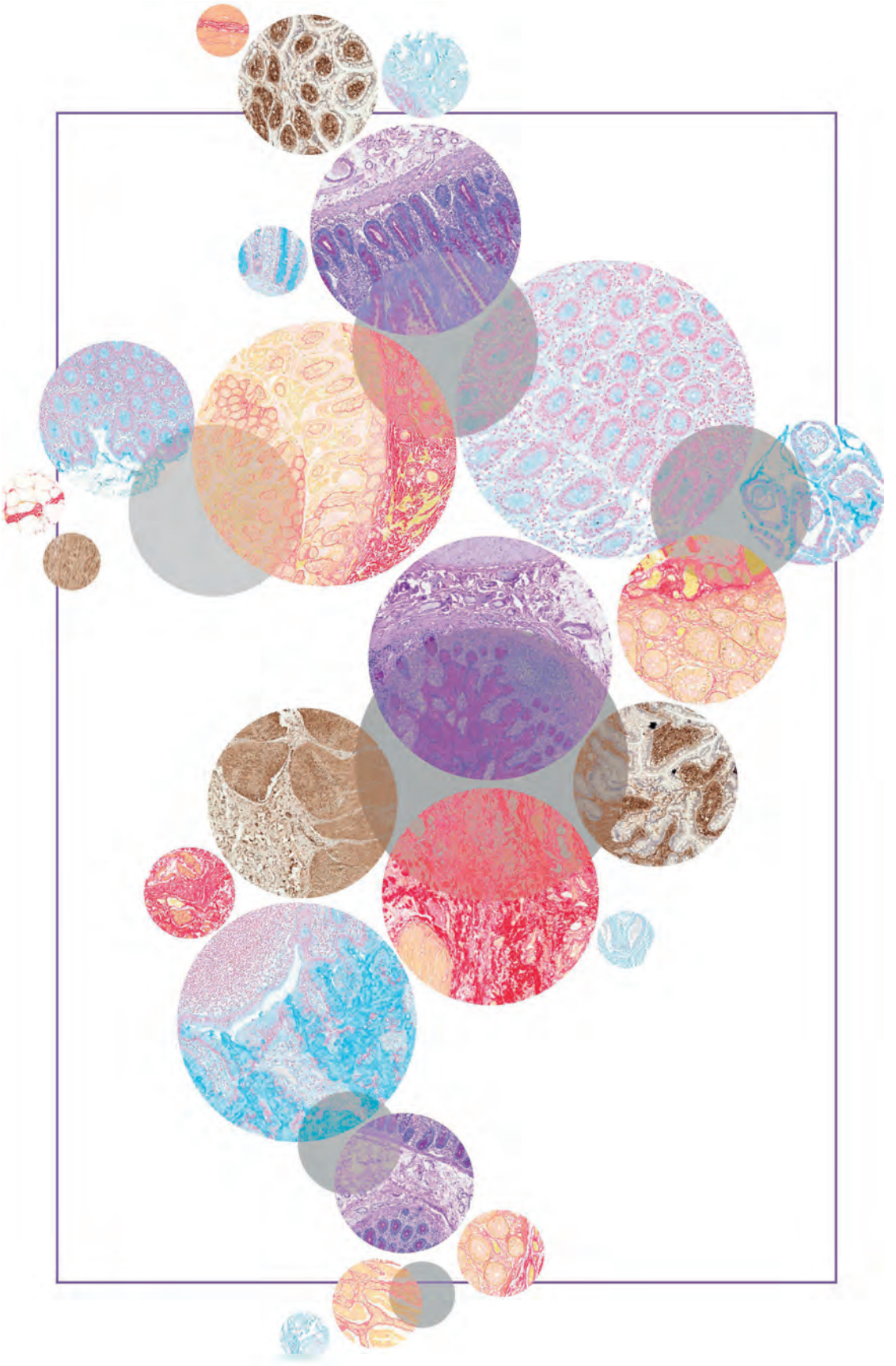


chapter ONE

Introduction

Inflammatory bowel disease (IBD) is a chronic immune-mediated disorder of the gastrointestinal tract. Considerable research efforts have been spent to understand the risk factors and biological mechanisms that drive gastrointestinal diseases. The most accepted hypothesis explaining its pathogenesis is, that environmental factors and gut microbiome trigger an exaggerated inflammatory response in a genetically susceptible individual. However, we still do not fully understand what is causing IBD.

In recent years, the development of new technologies provided the research community with advanced tools to uncover the yet hidden layers in the complexity of human biology. The work that I present in this thesis 'Gut mucosal gene expression in inflammatory bowel disease: the heterogeneous nature of inflammation' describes my approach to answering fundamental questions regarding the heterogeneity and complexity of IBD. The aim of this thesis is to gain insight into the genetic background and immune system traits of patients with IBD in order to reveal potential novel therapeutic options. This research focuses on the tissue where the disease naturally occurs – the human gut – and makes use of new sequencing technologies, such as single-cell RNA sequencing, to acquire highly detailed information.

CLINICAL DEFINITION OF IBD

IBD is an overarching term for two diseases: Crohn's disease (CD) and ulcerative colitis (UC). Patients with IBD experience recurrent abdominal pain, disabling defecation patterns and low energy levels. Both diseases are characterized by inflammation of the gastrointestinal tract and can be accompanied by extra-intestinal manifestations, such as arthritis and uveitis. The peak incidence runs between the age of 20 and 30 years, and some studies report a second peak at the age of 50-60 years[1,2].

Clinical definition of Crohn's disease

In the Netherlands, CD has an estimated prevalence of 2-3 per 1000 persons, with a slightly higher prevalence in women[3]. The inflammatory pattern is patchy, and can occur throughout the entire gastrointestinal tract. On endoscopy and radiological imaging, the typical findings for CD include a thickened bowel wall with deep ulcers and fissures in the mucosa. Aphts and fistula are often present. Microscopically, CD is characterized by transmural inflammation and, in some cases, granuloma formation **(figure 1).**

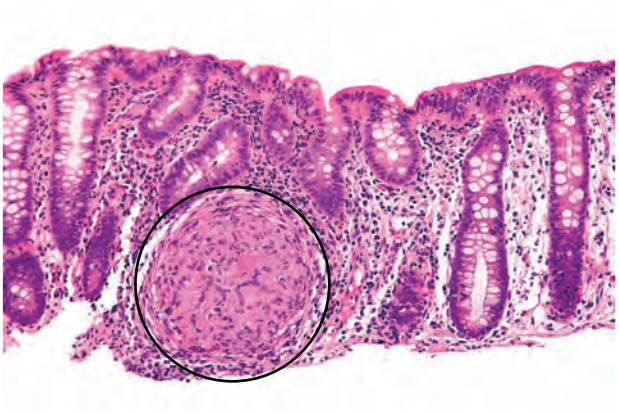


Figure 1. Histopathological image of CD colon with granuloma formation[4]

Clinical definition of ulcerative colitis

The estimated prevalence of UC is 4 per 1000 individuals[5]. In contrast to CD, the inflammatory pattern is sharply delineated and generally starts from the most distal, left part of the colon. The endoscopic image typically shows a reddened, inflamed colon, with erosions or ulcerations that bleed easily. Microscopically, the inflammation is restricted to the mucosal layer, and loss of goblet cells can be seen **(figure 2)**.

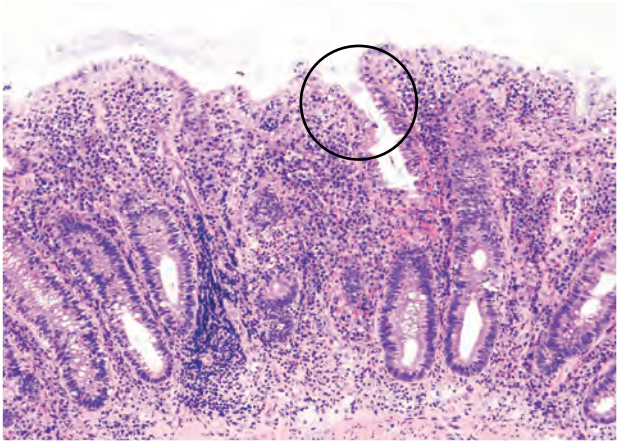


Figure 2. Histopathological image of UC colon showing loss of goblet cells[5]

IBD is a heterogeneous disease. While approximately 90% of the patients with IBD can be classified as CD or UC based on their disease characteristics, in roughly ten percent of IBD cases the disease pattern does not fit the definition of either[6–8]. Instead, these patients are diagnosed with IBD-undetermined (IBDU), a disease entity that highlights one of the gaps in our knowledge of IBD pathobiology.

PRIMARY SCLEROSING CHOLANGITIS: A RISK FACTOR FOR IBD

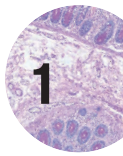
Primary sclerosing cholangitis (PSC) is a chronic inflammatory liver disease characterized by sclerosis of the bile ducts. Around 3% of patients primarily diagnosed with CD and 5% primarily diagnosed with UC develop PSC. Of all patients with PSC, approximately 75% have concomitant IBD, underlining the connection between the two diseases[9].

Although only a small part of patients with IBD develop PSC, PSC-IBD presents with distinct clinical characteristics and, therefore, is discussed here as a separate entity. For instance, in contrast to UC, the inflammation in PSC-IBD patients is generally located in the right side of the colon, is relatively mild and shows frequently rectal sparing. Furthermore, the risk to develop colon cancer is up to 5 times higher in PSC-IBD, than in "regular" IBD[10]. To date, little is known about the cellular and molecular background of PSC-IBD.

CURRENT TREATMENT IN IBD

There are various therapeutic options to treat IBD. Prednisolone, for example, can be used as a first step, for remission-induction. Aminosalicylates (e.g. mesalazine) are prescribed for maintenance therapy. In case of persistent inflammation, thiopurines (such as azathioprine), biologicals (such as infliximab) and small molecules (such as JAK inhibitors) are prescribed. Within the group of biologicals, many drugs have been developed in the recent years. For example: novel tumor necrosis factor alpha (TNFα)-inhibitors, that bind the cytokine TNFα, vedolizumab, that binds a cellular integrin, and ustekinumab, that inhibits interleukins IL-12 and IL-23. Furthermore, several small molecules, mostly influencing (immune) cell migration and communication, are in trials or in use for IBD. Examples are SMAD7 antisense oligonucleotides, sphigosine-1-phosphate (S1P) receptor modulators and phosphodiesterase (PDE)4 inhibitors.

Despite the extensive list of IBD medication, one of the current problems in the management of IBD is the lack of a treatment guaranteeing long lasting remission. It has been shown that biologicals are effective in only 30%-40% of patients, rendering a heavy burden for both patient's wellbeing and health care costs[11–13].
Clinicians face the challenge of choosing the right treatment for each patient, requiring decent clinical therapy decision tools. To establish these, there is an urgent need to understand the working mechanisms behind these drugs and the mechanisms underlying the response to therapeutics in IBD patients.



EXOGENOUS PLAYERS IN PATHOGENESIS OF IBD

In Europe and North America, the incidence of IBD seems to have reached a plateau, whereas in Asia, Africa and South America, disease incidence is still growing[14]. Besides heterogeneity in genetic background, environmental exposures such as industrialization, urbanization and rise of cigarette smoking are likely to underlie these regional differences[15]. Moreover, a relation between IBD and the introduction of modern dietary patterns (e.g. fast food), has been observed in the countries that show a rise in the IBD incidence[16]. In particular gut microorganisms are thought to play a role in this process.

The exposome and IBD

Epidemiological studies have identified lifestyle patterns and environmental factors that drive gastrointestinal diseases, the so-called 'exposome'[17]. Cigarette smoking, for example, has been associated with both developing as well as aggravating CD. In contrast, smoking cessation is a known risk factor for developing UC. Furthermore, the use of Non-Steroids Anti-Inflammatory drugs (NSAIDs) and the use of antibiotics in early life may increase risk of IBD later in life. On the other hand, living in the countryside is protective for both UC and CD, as are vaginal birth and breastfeeding[18,19]. In recent years, dietary patterns have been investigated for their potential effects on disease development. More specifically, the sugar-rich and high-fat Westernized diet has been suggested to have inflammatory effects. Conversely, Mediterranean and fiber-rich diets have been investigated for anti-inflammatory properties and their potential for the prevention of inflammatory disease[18].

Gut microbiota composition and its associations with IBD

Microorganisms that reside in our body form ecosystems, so-called 'microbiota'. The evidence for a role of the gut microbiota in the pathogenesis of IBD is accumulating, although it has been difficult to establish exact mechanisms of interaction with the host. The main characteristics of IBD-associated microbial changes include the decrease in bacterial richness and the depletion of strict anaerobes in combination with the blooming of facultative anaerobes[19].

Lately, it has been shown that the microbiota aid in metabolizing drugs, such as the antacid pantoprazole and the antidepressant fluoxetine[22]. This suggests that the microbes may not only directly contribute to the pathology of IBD, but may also influence treatment efficacy.

In addition, other members of the gut ecosystem such as viral species ('virome') and fungal species ('mycobiome') have been shown to be involved in IBD[23]. Therefore, microbiome research (*i.e.*, the study of microorganisms and the genes they express) may hold important answers for the development of treatments and uncovering disease pathology.

GENETIC VARIATION PREDISPOSING TO IBD

Studies in genetically identical twins identified IBD as a heritable disease in the 1980s[24]. Unlike for highly heritable, monogenic diseases such as cystic fibrosis, defining a causal gene for less genetically penetrant diseases such as IBD proved to be a complicated task. Large genome-wide association studies (GWAS) discovered that IBD is not mediated by a single genetic variant, but is instead a complex disease, mediated by a combination of multiple genetic variants, such as single nucleotide polymorphisms (SNPs), in combination with environmental, immunological and microbial factors. International collaborations between IBD research groups have resulted in the identification of more than 240 genetic risk loci associated with IBD[25]. These risk loci are classically involved in autophagy (e.g. *ATG16L1* and *IRGM*), microbial handling (e.g. *NOD2*), and T cell signaling (e.g. *IL23R*) pathways. However, for many associated risk loci, their route of effect remains unknown.

While early genetic research aimed to associate genetic variations to the presence of IBD, the current focus of research has shifted to inferring the underlying disease pathways and the causal effect of genetic variation on disease through, for example, gene expression studies. To understand the functional effect of a genetic variant, the relation between gene expression and genetic variants is studied in expression quantitative trait loci (eQTL) studies. In other words, an eQTL is an effect of genetic variants on gene expression. eQTLs may be identified by correlating a large dataset of genetic variant data to gene expression data derived from the same individuals **(figure 3)**.

Due to technical challenges, most studies are limited to investigating gene expression in blood cells only. Recently however, the Genotype-Tissue Expression (GTEx) consortium has released tissue-specific genetic expression data from several organs[26]. This has allowed the research community to extrapolate previous findings to specific, (disease-)relevant tissues. This database was used as a reference for studies that are featured in this thesis. Even though the GTEx database is a great resource, it is important to state that its data mainly originates from general population post-mortem samples, and the database is therefore less suitable to study disease-specific biology.

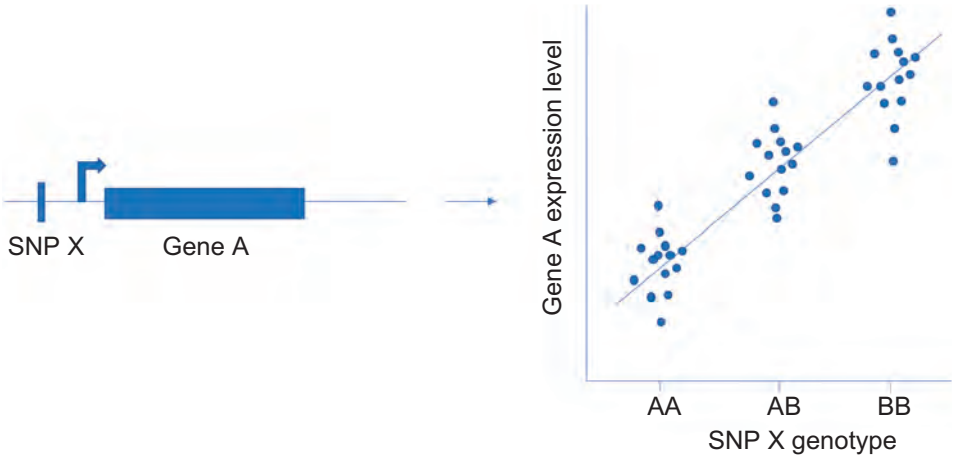


Figure 3. eQTL: a Single Nucleotide Polymorphism (SNP X) influences gene expression of Gene A in a linear fashion, depending on genotype

GUT TISSUE COMPONENTS AND THE IMMUNE SYSTEM IN IBD

The gut is a complex organ, which, next to nutrient intake and fluid organization, plays an important role in defense against exogenous exposure. Moreover, it contains the largest immune compartment of the human body[27] that defends against, and at the same time provides tolerance to, commensal microorganisms. In IBD, the tolerance mechanism is impaired and, as a consequence, an exaggerated immune response takes place. This inflammatory response can be largely divided into the initial innate immune response, and the secondary adaptive immune response. The innate immune response is mediated by cells, such as macrophages and dendritic cells, that a-specifically recognize the non-self. These cells can provide an initial and rapid response to pathogens, and clear the threat through, for example, phagocytosis. By releasing signaling cytokines, innate immune cells communicate with the adaptive, highly specific immune system, consisting of lymphocytes: T cells and B cells. These cells harbor memory for immune reactions to enable a quicker and more specific response to threats that challenge the host more than once (**figure 4**).

Gut tissue and its lines of defense

Being on the border with the outside world, the gut has an important role in border control. The mucus shapes the first line of defense against potential pathogens in the gut: it physically separates bacteria and food components from the gut wall. Under homeostasis, it prevents pathogenic microbes to enter the intestine, while it allows nutrients to pass[28]. External factors, such as bile salts, may influence the absorptive function of this layer[29], suggesting a link between liver and gut diseases.

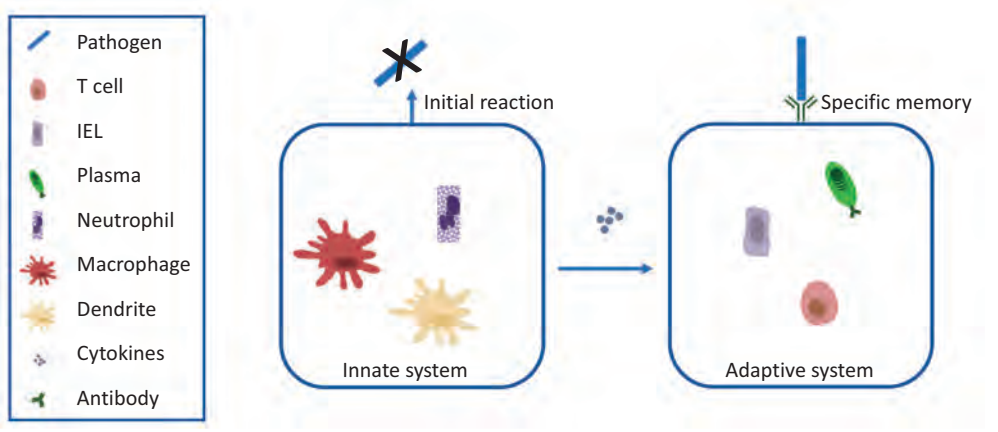


Figure 4. Innate versus adaptive immune response. The innate immune system a-specifically recognizes a pathogen, signals the cells from the adaptive immune system that create specific immune memory against pathogens. IEL: intraepithelial lymphocyte

The second layer of defense is formed by secretory and absorptive epithelial cells and intra-epithelial lymphocytes (IELs). The epithelial cells were previously thought to form a physical barrier only, while IELs would effectuate the actual immune reaction against unwanted intruders. However, recent research has shown that epithelial cells also contribute to the immune response through immune cell properties, such as antigen presentation[30].

The third line of defense is mediated by the lamina propria, which hosts various immune cells and builds a connection between the gut and the inner blood environment. The lamina propria consists of a wide variety of cells, including innate immune cells, vascular lining, fibroblasts and neuronal cells, all contained within a structure of collagen and other matrix proteins (**figure 5**).

Innate immune cells

In healthy mucosa, macrophages do not produce cytokines in response to a large variety of triggers, but at the same time retain their phagocytic and bactericidal functions. In IBD, it is hypothesized that the antibacterial function is impaired and that macrophages acquire an inflammatory state. Gut dendritic cells may have both inflammatory and tolerogenic properties, and play a role in the interaction with other cell types such as lymphocytes, epithelium and stroma. Upon active disease, neutrophils invade the gut epithelium where they play a dual role in both initiating and resolving inflammation[31].

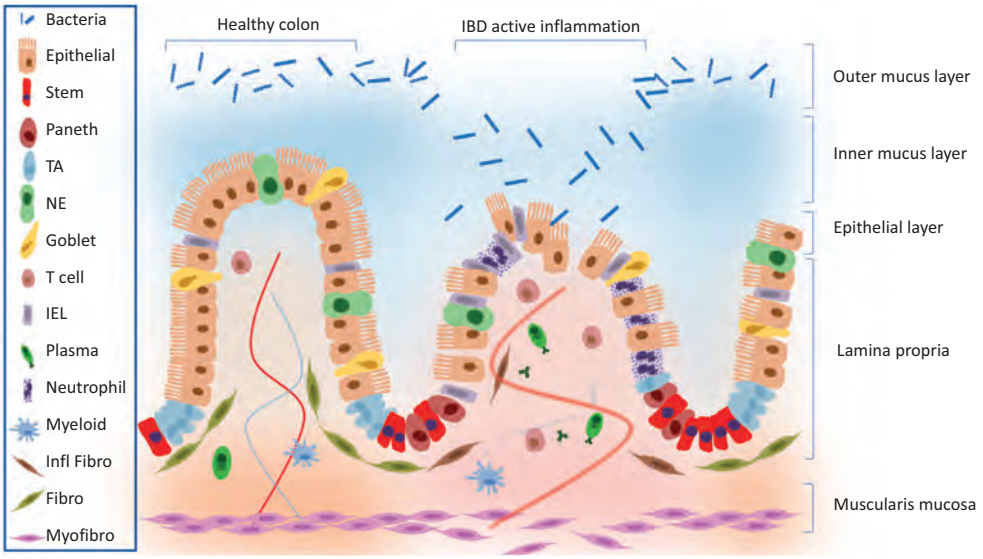


Figure 5. The schematic cellular composition of a healthy colon crypt vs an actively inflamed crypt in IBD. The healthy crypt is marked by nicely organized crypt cells and an intact fibroblast layer. On the left a legenda of cell types. TA: transit amplifying, NE: neuroendocrine, IEL: intraepithelial lymphocyte, (Infl) Fibro: (inflammatory) fibroblast, Myofibro: myofibroblast

Adaptive immune cells

The adaptive immune response is shaped by T and B cells. There are three main types of T cells: T-helper cells (Th) support the immune response by activating cytotoxic T cells (CTL), which remove intruders, while regulatory T cells (Tregs) help distinguish self from non-self. In turn, B cells can be divided into three classes: 1) a naïve parent B cell, expressing antibodies; 2) activated B cells, which are primed with antigen and further differentiate into plasma cells secreting antibodies; and 3) memory B cells, harboring memory for the encountered antigen.

Classically, IBD was defined as a T cell-mediated disease: UC was thought to be driven by the overactive Th type 2 (Th2) cells that react to triggers by producing interleukin-13; whereas CD was thought to be characterized by a disruption of mainly Th type 1 (Th1) cells, producing interferon-gamma (IFNy), and Th type 17 (Th17) cells, producing interleukin-17[32,33]. Recently, a broader and much more nuanced involvement of immune cells and immune responses in both diseases has been identified. B cell activation, for example, is implicated in both UC and CD, with higher antibody titers linked to a more severe clinical disease course. This supports the theory that microbial triggers are involved in the disease process[34]. Although the function of Tregs has been well-characterized in murine IBD-like disease, their role in human IBD is yet to be established[35].

RECENT DISCOVERIES ON CELL TYPES IN IBD INFLAMMATION

Current findings support the theory that not only immune cells, but also epithelial, endothelial and mesenchymal cells shape IBD pathology[36,37]. Several mechanisms of their involvement have been described. First of all, in-vitro studies demonstrated that epithelial cells can present antigens to $CD8^+$ T immune suppressor cells from the blood, a cross-talk that is lost in IBD-derived epithelial cells[38] **(figure 6-1)**. Secondly, sustained dysfunction of the mucus-secreting subtype of epithelial cells, the goblet cells, can lead to increased permeability of the mucus layer, thus making the gut epithelium easily accessible to bacteria **(figure 6-2)**. Thirdly, overactivation of myofibroblasts may cause defective repair, resulting in fibrogenesis[39,40] **(figure 6-3)**. Moreover, endothelial cells seem to be involved in microvascular inflammation induced by platelets[41] **(figure 6-4)**. Lastly, IFNγ production by T cells, as occurs in IBD, has been shown to induce apoptosis of intestinal stem cells in mice **(figure 6-5)**, a mechanism which may impair gut epithelial healing after a period of inflammation. Altogether, these findings indicate that there is a vast interplay between all different cell subtypes in the gut mucosa that shapes the IBD pathology[42].

In summary, a broad collection of factors, amongst which the exposome, microbiota and genetic alterations, is associated with a multi-cell-type-based inflammatory reaction in the intestine in patients with IBD. The interactions between these factors have been subjected to research in an attempt to distinguish those that are causal to, from those that are a consequence of the disease. Developments in lab techniques and bioinformatics are on the way to help us in this dilemma.

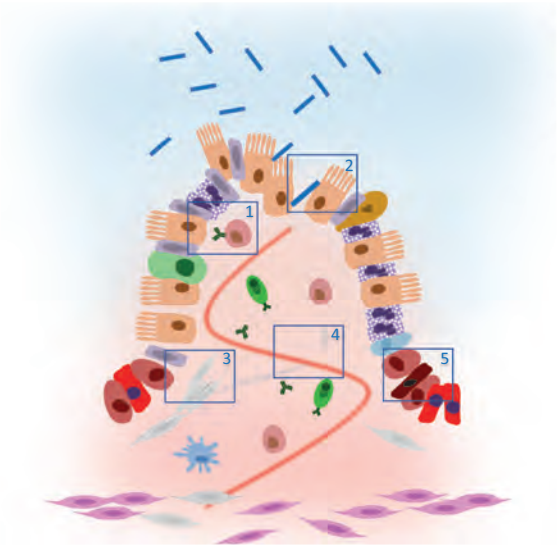


Figure 6. Mechanisms in IBD inflammation. The inflamed gut shows: loss of epithelial antigen presentation **(1)**; easy access to bacteria **(2)**; overactivation of myofibroblasts resulting in fibrogenesis **(3)**; microvascular inflammation induced by platelets **(4)**; apoptosis of intestinal stem cells **(5)**.

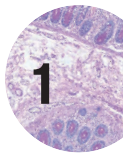
TECHNIQUES FOR STUDYING CELL TYPES INVOLVED IN IBD

Our current knowledge of cell biology stretches only as far as our technologies for studying cell and tissue characteristics allow. For example, a classical technique that is used in cell biology to infer expression of a protein is an antibody staining on histological slides, which is usually limited to detecting one or two signals at a time. In turn, technologies such as fluorescence-activated cell sorting (FACS) enable us to study the functional properties of multiple (tissue) cell types in one experiment. Defining the multitude of cell types and their interactions, however, has proved difficult.

Previously, most cell types in the human body were characterized based on their location, morphology and/or their extracellular receptors. Today, as messenger ribonucleic acid (mRNA) sequencing of single cells (scRNAseq) arises, cell type definitions have expanded, and with that our understanding of the cell diversity in the human body has increased. Cells can now be characterized by their transcriptional profile, allowing us to translate the cellular expression pattern into cell function, cell-cell interactions, co-expression and cell origin and fate characteristics. This development has enriched cell type definition from only surface marker-based to including mRNA clustering-based cell typing. An example that perfectly illustrates the change in the cell biology paradigm is the Human Cell Atlas project: a collaborative global effort with the aim of describing and redefining cell types of the human body, with their functions and their interactions based on scRNAseq experiments. The concept of a typical scRNAseq experimental workflow is illustrated in **Figure 1** of **chapter 9**.

COHORTS USED IN THIS THESIS

The studies described in this thesis make use of samples generously donated by participants of the Parelsnoer IBD cohort, the 1000IBD cohort and the GEID study. The Parelsnoer IBD cohort is a Dutch national collaborative biobank that prospectively collects clinical data and tissue samples of roughly 3000 patients with IBD, facilitating clinical and epidemiological research and enabling genotype-phenotype research in IBD[43]. 1000IBD is a cohort from the University Medical Center Groningen (UMCG, The Netherlands) that includes more than 1000 IBD patients and provides various layers of clinical, genetic, genomic, transcriptomic, proteomic, diet and other environmental data, aiming to discover molecular subtypes of IBD[44]. The GEID study, which has enrolled roughly 400 patients, aims to determine molecular mechanisms that drive IBD and to study how these differ from other gastrointestinal inflammatory diseases. At the same time, it aims to cover the functional follow-up of molecular insights. Each of these cohorts provides the valuable possibility to link clinical data (e.g. disease course, medication use and response to the treatment) to molecular characteristics, thus enabling development of prediction models and design of molecular disease-phenotypes.



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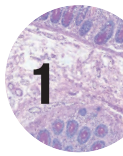
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THESIS OUTLINE

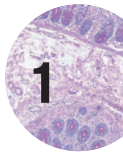
This thesis starts with an introduction to the genetic background of IBD and the research developments over the past decades in **chapter 2**: from targeted gene studies to whole genome sequencing. The chapter highlights the main findings from genetic studies in IBD and discusses the development of methods to identify genes and genetic variations that are not only associated with, but also causal to the disease. This brings us closer to uncovering the true IBD pathology and to envisioning targeted treatments.

We took a step towards finding causality of genetic variants with **chapter 3**, by linking known genetic variations with bulk RNA sequence data from the gut mucosa of IBD patients. We show the influence of these variations on gene expression, potentially protein expression and possibly on (disease) pathways. Next, we researched whether these genetic effects on gene expression depend on the inflammation status and cell composition of the gut mucosa.

Gut tissue harbors many different cell types. Within human gut mucosa, T cells are classically considered to be main players in IBD pathology. Which T cell subtypes have a key role in IBD, why and how are questions that are subject to a continuous debate. A potent emerging technology that can help us find answers is scRNAseq. In **chapter 4**, we used this method to study the T cell subtypes in terminal ileum mucosa, that are involved in CD. To point out which cells contribute most to disease pathology, we compared the obtained gut T cells to T cells deriving from the peripheral blood of patients with CD. Furthermore, we aimed to identify drugs, prescribed for other diseases than IBD, that may have potential for repurposing.

Since the inception of scRNAseq, studies that employ this technology have deepened our knowledge on gut tissue homeostasis. **Chapter 5** summarizes the most significant discoveries made by scRNAseq in human gut biology. The chapter summarizes both gene expression and cell type composition across various gut diseases, such as celiac disease, colorectal cancer and IBD.

A crucial step in the scRNAseq workflow is dissociation of the tissue into single cells. Even though scRNAseq is known to be sensitive to perturbations, different dissociation protocols are used for mucosal tissue dissociation in IBD research. To provide an overview of the individual effects of dissociation techniques on gene expression, we compared the influence of three different gut mucosa dissociation protocols on single cell gene expression in **chapter 6**. Because current methods are based on fresh gut mucosal biopsies and thereby limited by logistics, we investigated the use of cryopreservation to



enable large-scale studies. We summarized our findings in a decision tool to help fellow researchers with choosing a suitable experimental design.

Although treated like IBD, the clinical aspects of PSC-IBD suggest that different disease-specific mechanisms are involved, which to date remain largely unknown. In **chapter 7**, we investigated inflamed and non-inflamed mucosa from patients diagnosed with either PSC-IBD or UC, and from healthy individuals. We searched for distinct patterns that differentiate PSC-IBD from UC, which may in turn explain differences in clinical disease behavior.

A discussion of the findings presented in this thesis may be found in **chapter 8**, and future directions in IBD research are suggested in **chapter 9**.