

MUCOSAL CYTOKINE PROFILES AS BIOMARKERS IN INFLAMMATORY BOWEL DISEASE

Translating molecular biology into clinical medicine



Renathe Rismo

A dissertation for the degree of Philosophiae Doctor





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by

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Naturvitenskapens oppgave er ikke bare å utvide erfaringa, men å få en orden i denne erfaringa.

~Niels Bohr~

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Norsk sammendrag-Norwegian summary

Patogenesen ved inflammatorisk tarmsykdom (IBD) er fortsatt ikke fullstendig klarlagt. Mye forskning har blitt gjort de siste tjue årene for å kartlegge immunresponsen ved ulcerøs kolitt og Crohns sykdom. Man antar at et samspill mellom genetiske faktorer, miljøfaktorer og bakterier ligger bak utviklingen av sykdommen. Kartleggingen av immunresponsen i tarmen har vært grunnlaget for utviklingen av såkalt målrettet behandling, også kjent som anti-TNF behandling.

I denne avhandlingen er sammenhengen mellom uttrykket av immunkomponenter i tarmslimhinnen, respons på behandling og sykdomsforløp undersøkt hos pasienter med IBD. Arbeidene er basert på innsamlet pasientmateriale ved gastromedisinsk avdeling UNN Tromsø i perioden 2003-2011. Det er gjort kliniske registreringer i tillegg til innsamling av vevsprøver fra tarmen under endoskopiske undersøkelser av disse pasientene.

Vi har først undersøkt immunprofilen i tarmen hos IBD pasienter som er uten behandling og funnet at enkelte immunmarkører er assosiert med grad av sykdomsaktivitet. Deretter har vi sett på om nivået av genuttrykk av noen markører kan relateres til behandlingseffekten. Vi har funnet at det hos pasienter med ulcerøs kolitt ser ut til at et høyt uttrykk av enkelte mediatorer før oppstart av anti-TNF behandling kan forutsi en bedre effekt av behandlingen. Hos pasienter med Crohn har vi sett at anti-TNF behandling reduserer uttrykket av immunmarkører som er relatert til betennelsen, men ikke til normalisert nivå hos de fleste pasienter. Manglende normalisering av uttrykket av markører som medierer betennelsen hos Crohn-pasienter gir signifikant høyere risiko for raskt tilbakefall når anti-TNF behandlingen stoppes.

Immunmarkører som relateres direkte til betennelsen i tarmslimhinnen hos IBD pasienter ser ut til å kunne utnyttes som biomarkører i klinisk sammenheng ved å forutsi både effekten av behandlingen og sannsynligheten for raskt tilbakefall etter behandlingen er stoppet. Videre kartlegging av immunrespons og identifisering av verdifulle biomarkører er viktig for å utvikle målrettet individuell behandling, og også definere kriterier for valg av behandlingsstrategi på lang sikt.

English summary

The pathogenesis of inflammatory bowel disease (IBD) is still debated. Extensive research has been performed over the past 20 years to reveal mechanisms driving the immune response in the intestine of patients with ulcerative colitis (UC) and Crohn's disease (CD). The main hypothesis is that an interaction between genetic, environmental and microbial factors is involved in the development of the disease. Characterization of the intestinal immune response has set the foundation for the development of targeted therapy or anti-TNF therapy.

In this thesis the association between gene expression of mucosal immune mediators, treatment response and disease course has been investigated in patients with IBD. The study is based on patients examined and followed at the Department of medical gastroenterology, UNN Tromsø, from 2003-2011. Clinical registrations and collection of intestinal tissue during endoscopic examinations of IBD patients have been performed.

First, we have examined the mucosal immune response in untreated IBD patients, and we have shown that certain immune mediators are positively associated with the grade of inflammation or disease activity. It also seems that the immune response does not differ between the two diseases. Next, we have shown that gene expression levels of certain mediators before initiation of targeted therapy in ulcerative colitis can predict the response to therapy. In patients with CD we have demonstrated that anti-TNF therapy reduces the gene expression levels of mediators identified as important players in the pathogenesis, but not to normalized levels in all patients. Finally, it seems that elevated gene expression levels of certain immune mediators in mucosa with healed appearance increases risk of early relapse after treatment cessation in CD patients.

Mucosal immune markers directly involved in the inflammatory process of IBD can potentially be utilized as biomarkers in daily clinical practice by predicting treatment response and long-term effect after discontinuation of therapy. Further characterization of mucosal immune response and identifying valuable biomarkers in IBD is important for the purpose of developing tailored therapeutic strategies.

List of papers

Paper I

Olsen T*, Rismo R*, Cui G, Goll R, Christiansen I, Florholmen J. TH1 and TH17 interactions in untreated inflamed mucosa of inflammatory bowel disease, and their potential to mediate the inflammation. *Cytokine* 2011;56:633-40.

*equal contribution

Paper II

Rismo R, Olsen T, Cui G, Christiansen I, Florholmen J, Goll R. Mucosal cytokine gene expression profiles as biomarkers of response to infliximab in ulcerative colitis. *Scand J Gastroenterol.* 2012;47(5):538-47.

Paper III

Rismo R, Olsen T, Cui G, Paulssen EJ, Christiansen I, Florholmen J, Goll R. The effect of adalimumab for induction of endoscopic healing and normalization of mucosal cytokine gene expression in Crohn's disease. *Scand J Gastroenterol.* 2012;Aug 6 (Epub ahead of print)

Paper IV

Rismo R, Olsen T, Cui G, Paulssen EJ, Christiansen I, Johnsen K, Florholmen J, Goll R. Normalization of mucosal cytokine gene expression levels predict long-term remission after discontinuation of anti-TNF therapy in Crohn's disease. (Submitted manuscript)

Abbreviations

APC- Antigen presenting cell
IBD- Inflammatory bowel disease
CD- Crohn's disease
CDAI- Crohn's disease Activity Index
DC- dendritic cell
FOXP3- Forkhead Box P3 (Treg transcription factor)
GATA3- GATA binding protein 3 (Th2 transcription factor)
IECs- Intestinal epithelial cells
IFNG- Interferon gamma
IFX- Infliximab
IHC-immunohistochemistry
IL- Interleukin
LP- Lamina propria
MH- Mucosal healing
NKT cell- Natural killer T cell
NOD- Nucleoide-binding oligomerization domain protein
RCT- Randomized controlled trial
RORC- RAR-related orphan receptor (Th17 transcription factor)
RT-qPCR- real-time quantitative polymerase chain reaction
STATs- signal transducers and activators of transcription
TBX21- T-box 21 (Th1 transcription factor)
TGFB- Transforming growth factor beta
Th cell- T helper cell
TLR- Toll-like receptor
TNF- Tumor necrosis factor (synonym: tumor necrosis factor alpha)
Treg- T regulatory cell
UC- Ulcerative colitis
UCDAI- Ulcerative Colitis Disease Activity Index

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1. Introduction

Inflammatory bowel disease (IBD) includes the two main phenotypes ulcerative colitis (UC) and Crohn's disease (CD). In addition 10-12% has an indeterminate form of colitis. UC and CD are chronic remittent diseases affecting the colon or potentially the entire gastrointestinal tract, respectively. The pathogenesis is complex and not fully understood, but genetic and environmental factors are most likely important for the development of the disease. In 2012 the most frequent proposed hypothesis is that IBD is the result of an aberrant immune response towards commensal bacteria.

1.1 Epidemiology and natural history of inflammatory bowel diseases

The epidemiology of IBD has been extensively studied, for more detailed review see [37,107,119]. The incidence peaks between 25-40 years and 15-30 years for UC and CD patients, respectively [37,199], and incidence appears to be increasing, especially for pediatric IBD and in low-incidence areas [3,189,201]. Norway is considered as a high incidence country [121,122]. A south-north gradient for incidence-rate has been demonstrated as well as an east-west gradient [18,174]. The incidence varies in different studies from 2-24/100 000 and 3-20/100 000 for UC and CD, respectively. Data on prevalence ranges from 40-500/100 000 and 50-322/100 000 for UC and CD, respectively. In the latest review [119] a statistically significant increase in the incidence of IBD was reported in 75% and 60% of CD and UC studies, respectively. It has been argued that UC is slightly more frequent in men and CD more frequently in women, but there are some diverging results on this, some also report an equal distribution among sexes. Higher incidence rate of IBD in urban areas and in areas with higher levels of education has been reported [2]. Environmental factors like hygiene, smoking, diet, vaccinations and infections, among others, have been associated with IBD development and prognosis [71,76,83,107,176]. Environmental and genetic factors in IBD development will be further discussed later in this thesis.

CD and UC are lifelong diseases. The disease course is unpredictable and characterized by periods of remission interrupted by relapses. The complications can be debilitating. In a recent study in south-eastern Norway the overall disability rate in the IBD population studied was nearly 20%, with a relative risk of disability pension around twice as large as the background population. The risk was highest in patients aged below 40 years [84]. Apart from surgery, lifelong pharmacotherapy has remained the cornerstone of IBD management. A breakthrough came in the early 1990's with the genetically engineered animal

models, identifying tumor necrosis factor (TNF; synonym TNF alpha) in the inflammatory process. This initiated the development of targeted biological therapy [143]. The following chapters of the introduction will provide some details on normal mucosal immune response, current hypotheses of IBD pathogenesis and basis for development of targeted therapy. A short overview of current therapeutic guidelines will also be given.

1.2 Normal intestinal immunity

The gastrointestinal system is a possible entry point for pathogens to access the internal environment of the body. Humans provide residence to numerous gut bacteria that contribute by shaping the molecular profile of our intestinal immune system. It is important that the intestinal immune system can distinguish between beneficial and pathogenic microbes. Normal intestinal immunity is comprised of fast-acting innate immunity and antigen-specific adaptive immunity, balanced by a regulatory component. Innate, adaptive and regulatory immunity is provided by several factors and immune cell groups found in the lining of the gastrointestinal tract. The epithelial cells that constitute the physical barrier also have immunologic functions. The following chapters will attempt to give an overview of the known characteristics and mechanisms of gut immunity.

1.2.1 Immune tolerance and intestinal homeostasis

The mucosal immune system maintains a balance between protective immunity and immune tolerance. The majority of antigens encountered by the intestinal immune system are not pathogens, but proteins from food and commensal bacteria [124,207]. This encounter should not normally induce an immune response. This state of unresponsiveness to antigen is called *oral tolerance*, and is believed to be based on the development of regulatory T cells (Treg), which is a result of tolerogenic stimulation by dendritic cells (DCs) in the gut [118,207]. We harbour more than a 1000 species of commensal bacteria in our intestine, mostly located in the colon, and mechanisms of immune tolerance are essential [32,118]. Intestinal epithelial cells (IECs) serve as a physical barrier to microorganisms and limit the activation of lamina propria (LP) immune cells, but a mechanism for tolerating intestinal microorganisms if the barrier is disrupted is still required. Prime determinant of oral tolerance is believed to be the dose of antigen involved, with low doses favouring the induction of Tregs and higher doses inducing anergy or deletion of the activated immune cells. It has been reported that intestinal commensal bacteria promote T cell hypo-responsiveness and down-regulate serum antibody responses induced by dietary antigen. A role of the liver has also been suggested, and

diversion of blood draining from the intestine to the liver by a portocaval shunt has been shown to impair oral tolerance [207]. Orally administered antigens are primarily recognized by DCs in the mesenteric lymph nodes. Tregs are then activated by the anti-inflammatory cytokines interleukin (IL) 10 and transforming growth factor beta (TGFB) [32]. Defects in certain subsets of Tregs have been observed in subjects with IBD, correlated with failure to induce oral tolerance in these patients.

It is, however, not fully understood how the intestinal immune system can discriminate between resident intestinal microbes and pathogens to which it must respond. Pattern recognition receptors (PRRs), described more extensively in chapter 1.2.2.2, do not distinguish between pathogens and commensal bacteria [32]. Other mechanisms are involved to avoid aberrant activation of the innate immune system. Both the physical barrier provided by the mucus, lower levels of PRR on the apical surface of intestinal epithelial cells and a modulation of signal transduction in host cells by commensals are thought to be involved in the lack of immune response towards these bacteria in the gut. There is, however, evidence that intestinal homeostasis is dependent upon responsiveness to the pathogen-associated molecular patterns (PAMPs, see 1.2.2.2) on commensals, and that intestinal immune cells are actively sensing these microorganisms [32]. Ultimately, virulence factors like secretion of membranolytic toxins, cell adhesion and invasion mechanisms, separate most pathogens from commensals [162].

1.2.2 Mucosal innate immunity

Most organisms capable of causing disease in humans are rapidly destroyed by the mechanisms of the fast acting innate immune system. The following sections will provide an overview of the current knowledge of intestinal innate immune response.

1.2.2.1 Intestinal mucosal surface, permeability and mechanisms of antigen uptake

The mucosal surface of the gastrointestinal tract is in continuous contact with food and microorganisms, and is a crucial site of immune regulation. Mucosal surfaces in general are lined by epithelial cells which establish a barrier between the external and internal environment, but are also selectively permeable, allowing exchange of fluids and nutrients [188,195]. A single epithelial cell layer with tight junctions lines the mucosa. A layer of mucus overlies the epithelium, and there is also a presence of associated immune cells. These components form the barrier and facilitate the adjustment to the exposure to microorganisms

[147]. The cells of the intestinal surface are the enterocytes, the goblet cells, the Paneth cells, endo-and phagocytotic M-cells specialized for antigen uptake, and intraepithelial lymphocytes [54,124]. Goblet cells are responsible for the mucus layer. The mucus layer consists of mucins that are secreted into the lumen and provides the important first line protection against bacteria. Part of the mucus layer is resistant to bacterial penetration [85,105,147,195]. Paneth cells are present at the base of the crypts throughout the small intestine, and are most abundant in the terminal ileum. They secrete mainly lysozyme and defensins, and express PRRs. It has been suggested that defective Paneth cells are involved in CD pathogenesis [163]. Damage to the impermeable IEC plasma membrane will result in loss of barrier function [195], and will be described in further detail later.

Antigen transportation across the epithelium is a strictly controlled process, and occurs primarily at the level of M cells [147]. Material is transported through the cell by transcytosis and released into the extracellular space at the basal membrane, in close proximity to lymphocytes and DCs. The DCs process and present the antigens to T lymphocytes. In addition, DCs can sample antigen directly by extending their dendritic processes through the epithelium without disruption of tight junctions [207]. Paracellular passage is regulated by a complex of proteins that form the tight junctions that bridge and seal adjacent epithelial cells. Tight junctions are crucial to the integrity of the epithelial barrier and can be further divided into occludins, claudins, tricellulins and junctional adhesion molecules. Regulation of assembly and maintenance of tight junctions is influenced by various stimuli. Commensal bacteria have, for instance, been shown to promote intestinal barrier integrity. In addition to bacteria, tight junctions are also regulated by dietary components (for review, see [196]).

1.2.2.2 Pattern recognition

Enterocytes express specialized receptors of the PRR-family, such as Toll like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors [118]. These are innate immune receptors that recognize PAMPs on microorganisms from the intestinal lumen, and are important for defence against intestinal pathogens [94]. Engagement of PRRs on mucosal cells activates downstream signalling cascades and promotes the production of pro-inflammatory cytokines and antimicrobial peptides, as well as maintenance of barrier function.

The most intensively studied PRRs are the TLRs. There are 10 different known TLRs in humans, located either on cell surface membranes or membranes on intracellular endosomes. The location is crucial to their function; some detect viral particles and others

extracellular microbes. TLR signalling in the intestine mediates functions that are crucial for keeping a healthy epithelial barrier, for example maintenance of tight junctions and secretion of anti-microbial peptides [100,163]. TLR4, for instance, is present on macrophages and detects lipopolysaccharide (LPS), a cell-wall component of Gram negative bacteria [124]. In inflammatory bowel disease an increased expression of TLR4 on IECs has been described [80].

The NOD-like receptors are another class of innate immune receptors (NOD1 and NOD2). They are located in the cytosol of IECs, macrophages and DCs, where they bind microbial products. They activate nuclear factor kappa B (NFkB) and initiate the same response as TLR engagement [94,124]. Activation of these receptors leads to increased cytokine production, increased chemokine production and cell-surface expression of co-stimulatory molecules that are essential for activating cells of the adaptive immune system. Polymorphisms of NOD2 are associated with Crohn's disease [173], resulting in impaired bacterial clearance (for review see [100]). NOD2 appears to be crucial for proper colonization of the intestinal microbiota [163].

1.2.2.3 Mucosal immune compartment and innate immune cells of the intestine

The extensive gut-associated lymphoid tissue (GALT) is the largest immune compartment in the body, located in the LP. Peyer's patches are aggregates of lymphoid tissue that are located in intervals in the LP of the intestine just beneath the epithelium. Immune responses in the intestine are initiated in the Peyer's patches. The lymphoid tissue comprises B cell follicles and T cells between the follicles. The subepithelial area overlying the patch is rich in DCs, T cells and B cells. Defects in the epithelial barrier may expose GALT to excess luminal antigens [173].

DCs and macrophages are abundant in the LP. Activation of innate immune signalling in DCs stimulates the secretion of cytokines and chemokines, and induces DC migration to mesenteric lymph nodes [94]. In the large intestine macrophages outnumber the DCs. Intestinal macrophages are highly phagocytic, but they do not release pro-inflammatory mediators and, unlike other tissue macrophages, lack the expression of the innate immune receptor CD14 [14]. Intestinal macrophages are thought to have a regulatory function, and produce large amounts of IL10 [32]. This is supported by the finding that depletion in animal models leads to intestinal inflammation. During inflammation the macrophage composition changes and the intestine is infiltrated with TLR responsive macrophages producing high levels of pro-inflammatory cytokines [14]. Kamada and co-workers identified a subset of

macrophages in the intestine of CD patients that expressed both macrophage markers and DC markers and produced large amounts of pro-inflammatory cytokines [91].

Another innate immune cell population in the LP is the innate lymphoid cells, with effector cytokine profiles resembling Th cells. Innate lymphoid cells promote containment of commensal bacteria to the intestine, thus preventing systemic spread (for review, see [85]). Neutrophils are a critical component of the early innate immune response to intestinal pathogens. They are rarely found in the healthy intestine, but increase rapidly in number during inflammation or disease. Natural killer (NK) T cells are a major component of the innate immune system. They are required for the first line of defense against infected cells and produce interferon gamma (IFNG), which makes them an important link between the innate and adaptive immune system [211]. The role of NK cells in IBD pathogenesis is still debated.

1.2.3 Adaptive immunity in the intestine

The T-cell population of the intestine consists of CD4⁺ and CD8⁺ T cell, in a ratio of about 3:1 in the LP. Intraepithelial lymphocytes are mainly CD8⁺ T cells that serve to maintain epithelial barrier function, respond to infection and regulate innate and adaptive immune responses. It has been reported that depletion of intraepithelial T cells can impair the induction of oral tolerance [172]. T cells secrete large amounts of cytokines with different effects when they are stimulated by antigen [124]. The following sections will provide some details on the T-cell mediated immune response in the intestine.

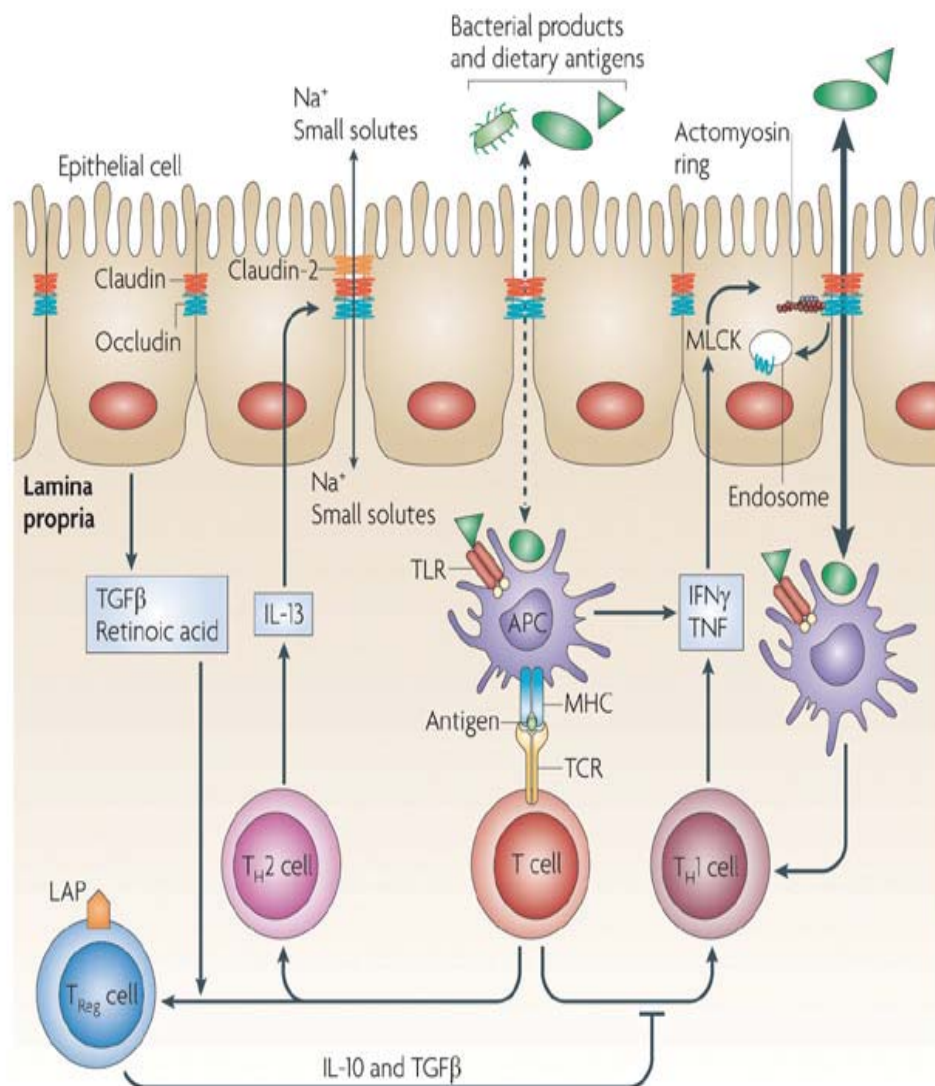


Figure 1 Mucosal immune homeostasis. Defects in the barrier may allow bacterial product to enter the lamina propria where it is processed by dendritic cells. This can lead to stimulation of effector T cells and a pro-inflammatory cascade or a stimulation of regulatory T cell response which maintains intestinal homeostasis [195].

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1.2.3.1 T cell activation and differentiation

T cell responses are initiated when a CD4⁺ or CD8⁺ T cell encounters an activated antigen-presenting cell (APC) that displays the appropriate antigen. This is called priming of naïve T cells and results in a phosphorylation cascade in the T cell. The differentiation is controlled by a set of cellular signals: signal 1, 2 and 3. Signal 1 is delivered through interaction between the T-cell receptor, the CD4 co-receptor and peptide:MHC complex. Signal 2 is initiated when a co-stimulatory molecule B7 on the activated APC engages CD28 on the T-cell surface, and is necessary for the clonal expansion of T cells. Signal 3 is provided by the cytokines produced by the APC and controls the direction of subset differentiation (see 1.2.3.2). Signal 1 and 2 triggers synthesis of cytokines and receptors such as IL2, the IL2-receptor which promotes differentiation into effector T cells, and also cytokines that direct the differentiation into the specific T cell subsets. This is further discussed in the section below. Naïve T cells recognizing self-peptides on cells that lack co-stimulatory factors found on professional APCs will be inactivated or become anergic. This provides important self-tolerance [124].

CD4⁺ T helper (Th) cells orchestrate adaptive immune responses to various microorganisms. They provide help for B cells during antigen-specific immunoglobulin production and promote wound healing [134]. In 1986 Mosmann and Coffmann introduced two distinct types of Th cells, based on characteristic cytokine production [120]. For the next 20 years it was accepted that CD4⁺ T-cells differentiated into these two subsets: IFNG-producing Th1 and IL4-producing Th2. Recently it became evident that the CD4⁺ Th-cell differentiates into at least 4 subsets (Table 1): Th1, Th2, Th17 and CD4⁺CD25⁺ Treg cells, defined by the different cytokine expression profiles [124,134].

Transcription factors are critical for a regulated Th cell differentiation, expansion and cytokine production. Each lineage requires two types of transcription factors: the signal transducer and activator of transcription (STAT) proteins, and the master regulators that are induced by the STATs. STATs and regulators act directly on cytokine genes to induce or regulate transcription. The essential factors for each lineage are listed in table 1. Inappropriate or uncontrolled regulation of the Th response to pathogens may lead to chronic infection or self-tissue damage [212]. The main determinant for Th cell differentiation is the cytokine environment at time of antigen encounter. The required cytokines will come from cells of the innate immune system which again will lead to activation of specific STATs and transcription factors (Table 1) [124,212].

1.2.3.2 Subsets of CD4+ effector T-cells and effector T cell plasticity

Th1 cells express the transcription factor T-box 21 (TBX21) and predominantly produce IFNG. Signal 3 in Th1 differentiation comprises the cytokines IL12 and IFNG. Signal 3 results in activation of an intracellular signalling pathway involving STAT1 and 4, which leads to expression of TBX21. This commits the cell to becoming a Th1 cell. Th1 cells activate infected macrophages through secretion of IFNG, thereby stimulating macrophages to secrete IL12 and amplifying the differentiation of Th1 cells [124,212].

Th2 cells are involved in the response towards parasitic infections, by production of IL4, IL5, and IL13. Signal 3 in the case of Th2 differentiation is delivered by IL4. IL4 activates STAT6, which again induces expression of the transcription factor GATA binding protein 3 (GATA3). GATA3 is a powerful activator of gene transcription of IL4, and also IL5 and IL13. Th2 cells inhibit the Th1 induced macrophage activation through production of inhibitory cytokines [124,134,212].

Th17 cells produce IL17A-F, IL23, IL21 and IL22 [212], and was recently recognized as a distinct effector lineage [79,140]. Commitment to the Th17 lineage is made when both IL6 and TGF β are present. This activates STAT3, which leads to expression of transcription factor RAR-related orphan receptor C (RORC) and production of the distinct cytokines. Immunity mediated by Th17 cells is particularly important at epithelial and mucosal surfaces, and overproduction of IL17 or unregulated Th17 response is associated with chronic inflammation [117,212].

Plasticity between the subsets of Th cells, however, is widely documented, and has raised the question of the relevance of dividing T cells into strict subsets. The classification of T cell subpopulations might be helpful in an immunological perspective, but not too relevant in a clinical setting. CD4+ T cells are able to change their profile of cytokine production, and also express more than one transcription factor (for review see [82,126,133]). Studies have shown that differentiated Th2 cells, for instance, can be re-programmed to adopt GATA3+TBX21+ and IL4+IFNG+phenotype [81]. A subset of GATA3-RORC double-positive cells have been identified and reported to be involved in the development of asthma bronchiale [204]. Th17 cells and Treg cells can both acquire the capacity to produce IFNG if exposed to certain stimuli. First, naïve T cells express both Treg transcription factor Forkhead box P3 (FOXP3) and RORC, in which an inflammatory environment would tilt the balance towards Th17 differentiation and IL17 production. Second, Th17 cells have the capacity to shift toward a Th1 profile, given specific cytokine stimulation (for review see [4,126,212]). It has been suggested that it may be more accurate to view the process of Th cell differentiation

as a result of varying ratios of transcription factors, which again is regulated by an array of extrinsic and intrinsic factors [133].

1.2.3.3 Regulatory T cells

The presence of Tregs in the intestine is important for maintaining intestinal homeostasis and immune tolerance [212]. Tregs modulate the intensity and duration of Th cell inflammatory response to ensure chronic inflammation does not occur [175]. Tregs express the transcription factor FOXP3, which is induced by the activation of STAT5. Tregs produce inhibitory or anti-inflammatory cytokines like IL10 and TGFB, dependent on the effector Th cells they are activated by [73,124]. It has been suggested that there are multiple populations of Tregs that utilize distinct mechanisms for suppression under different circumstances [89], which might explain somewhat conflicting results in different reports. Gut microbiota appears to be essential for the proper development of Tregs in the intestine [175].

Table 1 Main CD4+ T cell subsets, related factors and functions

Th cell subsets	Signal transducers	Transcription factors	Main cytokines	Main function in intestinal immunity
Th1	STAT1 STAT4	TBX21	IFNG IL12 TNF	Intracellular infection
Th2	STAT6	GATA3	IL4 IL5 IL13	Induction of IgE responses/allergy. Anti-parasitic immunity. Inhibits Th1 differentiation
Th17	STAT3	RORC	IL17A-F IL23 IL21 IL6 IL22	Extracellular bacteria and fungi. Important at epithelial and mucosal surfaces. Promotes local tissue destruction
Tregs	STAT5	FOXP3	IL10 TGFB	Maintaining immune tolerance and homeostasis.

1.2.3.4 B cells

The extracellular spaces are protected by the so called humoral immune response. Antibodies produced by B cells cause the elimination of extracellular microorganisms and prevent spread of infection. Th cells activate B cells that recognize the same antigen, which differentiate into antibody-secreting plasma cells and memory B cells. B cells can also act as antigen presenting cells to naïve T cells. Antibodies contribute to immunity by neutralizing pathogens, facilitating phagocytosis and activating the complement system. The dominant class of antibody in the mucosal immune system is IgA, which is produced locally by plasma cells present in the submucosal compartment. TGFB is abundant in the intestine and induces

isotype switch to IgA. IgA is secreted into the subepithelial space and transported across the epithelium by transcytosis to the luminal surface. Luminal or secretory IgA binds to the layer of mucus on the epithelial surface where it can neutralize toxins and prevent pathogens from adhering. Its main function is limiting pathogens from accessing the mucosal surfaces through a non-inflammatory first line defence [25,124].

1.2.4 Cytokines in the context of T helper cells

Cells of the immune system communicate with each other and other cells of the body through a class of small proteins known as cytokines. Their receptors activate direct signalling pathways that induce rapid changes in gene expression in the nucleus. As mentioned above, different cytokines are responsible for the differentiation towards the different subsets, and often suppress the development of other Th lineages [212]. For each Th lineage more than one cytokine is involved. The sections below will provide some details on cytokines that are either produced by or associated in other ways to the respective Th subsets.

1.2.4.1 Th1 associated cytokines: IFNG, IL12, TNF

IFNG is the signature cytokine of the Th1 subset, and is responsible for the cell-mediated immunity involved in intracellular infections. It has, along with other Th1 related cytokines, been associated with the pathogenesis of autoimmunity [45,117]. Another Th1 related cytokine is **IL12**, produced by activated antigen-presenting cells. IL12 induces the Th1 pathway [117,124]. **TNF** (previously TNF alpha) is a pro-inflammatory cytokine that play a pivotal role as an immediate effector of inflammation. It enhances IL12-, IL1-, and IFNG-production, has an anti-apoptotic role and acts in synergy with IL17 in stimulating pro-inflammatory processes [183,191]. It has been shown to increase intestinal permeability [188]. TNF exists in a soluble (sTNF) and transmembrane (tmTNF) form, both biologically active. It is expressed by most cells of the immune system and also other types of cells (neurons, smooth muscle) [191]. The major part of TNF is, however, produced by macrophages [205].

1.2.4.2 Th2 associated cytokines: IL4, IL5, IL13, IL33

IL4 is produced by Th2 cells. IL4 is required for IgE and IgG1 class switching in B cells and alternative activation of macrophages during parasitic infections. It also stimulates

differentiation of Th2, and stabilizes the Th2 phenotype [134]. IL4 is also thought to inhibit Th17 pathway and TNF [117]. **IL5** is another Th2 derived cytokine that mobilizes, matures and recruits eosinophils. Th2 cells also produce **IL13**, which induces goblet cell differentiation, mucus secretion and tissue repair [134]. Another IL13 producing cell is the NKT cell, shown to be involved in UC pathogenesis [68]. **IL33** is a newly described cytokine that is expressed mainly by epithelial and endothelial cells (for review, see [77]). It is up-regulated following pro-inflammatory stimulation, and induces Th2 cytokine production. It also activates basophils, mast cells, eosinophils and NKT cells. It is believed to be a potent activator of the innate immune system [116].

1.2.4.3 Th17 associated cytokines: IL17A, IL23, IL6, IL21

Production of the cytokines from the IL17 family (IL17A-F) comes mainly from Th17 cells, but to some degree from NK cells. **IL17A** is considered pro-inflammatory and acts in synergy with TNF to amplify the pro-inflammatory response to infection. Most parenchymal cells express IL17 receptors. Signalling through these receptors induces the production of pro-inflammatory factors such as IL6, IL1, TNF, IL8 and matrix proteinases. This results in tissue destruction and a positive feedback loop that commits naive T cells to the Th17 lineage [117,139]. IL17A also has a role in maintaining the mucosal barrier integrity by enhancing the synthesis of tight junction protein and strengthen the connection between ECs [95]. **IL23** together with **IL6** and **TGFB** are involved in promoting and stabilizing the Th17 phenotype. IL23 belong to the IL12 family and shares a subunit (p40) with IL12, both secreted by DCs secondary to PRR-derived signals [92] [202]. **IL21** is produced in large amounts by Th17 cells and amplifies Th17 cell differentiation [117].

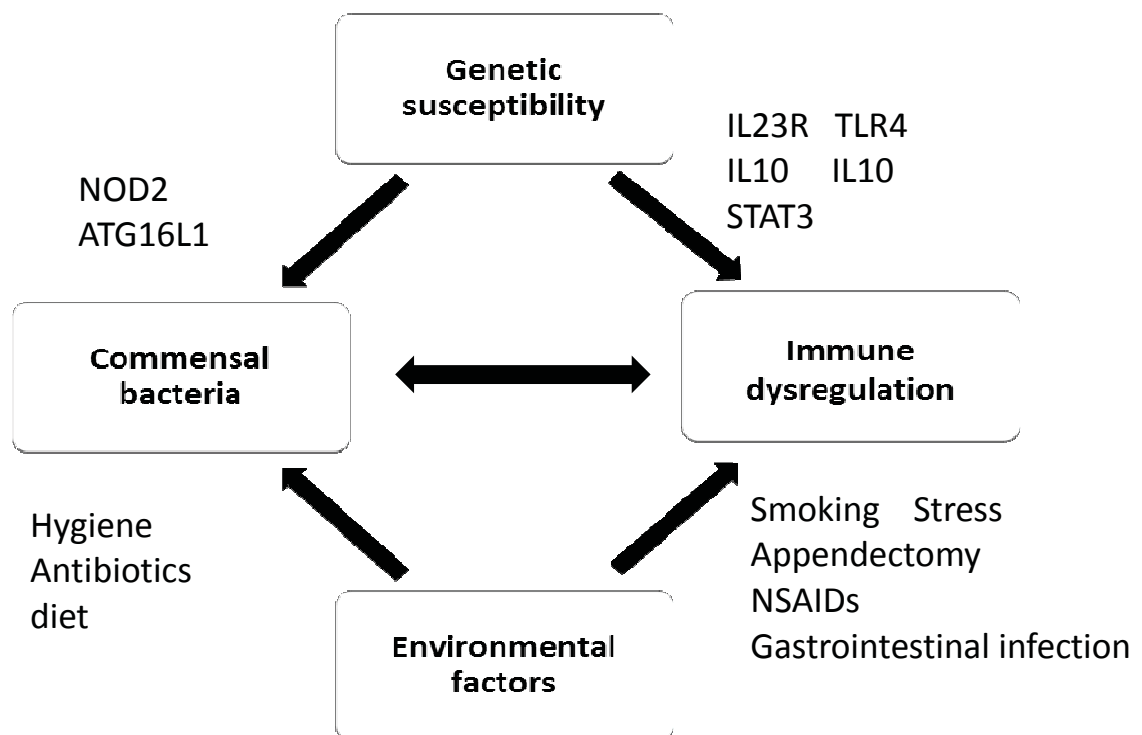
1.2.4.4 Treg associated cytokines: IL10, TGFB

IL10 and TGFB are the main cytokines arising from or related to the regulatory T cells. **TGFB** is a potent regulatory cytokine that inhibits T cell proliferation, differentiation and activation. It is produced by many different cells [22]. As mentioned, TGFB is involved in Th17 cell differentiation [117]. **IL10** secretion by Tregs controls mucosal inflammation. The development of spontaneous enterocolitis in *il10* knockout mice supports the involvement of IL10 in the mechanism of immune homeostasis [98]. IL10 may be produced by T cells, B cells, DCs, IECs, making the exact mechanism difficult to understand. IL10 signaling involves STAT3, which has been identified as a genetic risk locus for IBD [92].

1.3 IBD immunopathogenesis

Crohn's disease and ulcerative colitis are inflammatory conditions that are immunologically mediated. The leading hypothesis is that the onset is triggered by environmental factors in a genetically predisposed individual. The knowledge in this field is constantly growing; the following chapters will give a short overview of the current hypotheses of IBD immunopathogenesis.

Figure 2 Model of IBD pathogenesis



1.3.1 Genetic susceptibility and epigenetics

Familial aggregation of IBD has been reported since the 1930s. IBD patients have a first degree relative with the disease in approximately 20% of cases. Twin studies confirm the contribution of a genetic susceptibility with a concordance in monozygotic twins of 40-70% for CD and 10-20% for UC (for review see [15,92]). CD and UC are polygenic diseases and several genes involved in immunoregulation, mucosal barrier integrity and microbial clearance have been implicated. Genome-wide association studies (GWASs) have

significantly advanced our understanding of the genetics involved in IBD development and have also revealed a substantial overlap between CD and UC. As of 2011 almost 100 risk related loci or polymorphisms have been identified. Almost 30 polymorphisms are shared between CD and UC, and over 50% are also associated with other autoimmune or inflammatory conditions. Important genes associated with IBD are genes encoding NOD-like receptors in CD (*NOD2*), autophagy genes (*ATG16L1*, *IRGM*), genes encoding epithelial cell function and regulatory pathways, and genes involved in the IL23 and TNF signaling pathways which are involved in both forms of IBD [6,63,92].

Genetic susceptibility only explains around 20% of the heritability of IBD. For most human IBD susceptibility genes functional polymorphisms do not result in defects of the cellular immune system [6,63]. Evidence supports the theory that IBD is caused by a complex and abnormal interaction between genes and environmental factors. Heritable and reversible epigenetic alterations such as DNA methylation, histone modification and micro-RNAs may contribute to the disease pathogenesis by modifying the activation and function of genes, known as epigenetics. DNA methylation and histone modification are the most studied epigenetic mechanism. Numerous intestinal disease-associated DNA-methylations have been identified in IBD patients. In the study of Lin Z et al [104] differential changes in the methylation state of IBD-associated genes were significantly associated with UC and CD, potentially contributing to the onset and progression of the disease. Finally, micro-RNAs are involved in gene activity by post-transcriptional regulation of gene expression. Micro-RNA is a single-stranded, non-coding RNA molecule acting as a potent negative regulator of mRNA abundance and translation. There is increasing evidence that micro-RNA may have effects on the innate and adaptive immune response in several autoimmune diseases including UC and CD. Differential expression of micro-RNAs has been detected in active and inactive UC, and between ileal and colonic CD [44]. This is a scientific field in its early beginning, and further details are beyond the scope of this thesis. However, the potential of micro-RNA alterations to induce dysregulated immune response and be responsible for the pathogenesis of IBD is quite obvious. In general, the genomics as reported above show that IBD is a disease with a strong but very heterogeneous genetic predisposition. However, there is a lack of functional genomic studies to define how the genetics are coupled to the inflammatory process of IBD.

1.3.2 Environmental factors and role of the microbiota

A family history of IBD is the most important independent risk factor of IBD. Environmental factors in IBD development are also very important. There is, for instance, a considerable

amount of discordance in monozygotic twins. The development of IBD increases in immigrants to the western countries and an increasing incidence in low-incidence countries undergoing rapid westernization is reported. In general, the environment can influence gene expression by epigenetic factors as described above. Moreover, as mentioned in section 1.1 the incidence in northern Europe is higher compared to southern parts of Europe, and in addition there is an accumulation in urban areas compared to rural. Several environmental factors have been studied; an extensive description is, however, beyond the scope of this thesis.

Intestinal microbiota is likely the most important environmental factor in IBD. Humans harbour more than 10^{14} microorganisms from more than a 1000 species. Over 90% belong to the Bacteroidetes (Gram negative bacteria) and Firmicutes (Gram positive bacteria). The microbiota is required for the proper development and differentiation of the intestinal immune system, and normally equilibrium between tolerance and responsiveness is maintained (for review see [92,207]). Commensals compete with pathogens for space and nutrients and inhibit pro-inflammatory signalling responses. In addition they produce essential cofactors such as vitamin K and fatty acids [118,124]. Hygiene, socio-economic status, age, diet and lifestyle will influence the composition of the gut flora (for review, see [166]). Studies have revealed a detectable difference between the intestinal microbiota in IBD patients and healthy controls, with a lower biodiversity and lower bacterial load in the gut of IBD patients [62]. However, the dysbiosis is not necessarily causal, but may result from the conditions in an inflamed gut. Unrestricted immune response towards these commensals will lead to inflammatory bowel disease, discussed in the section below.

1.3.3 Abnormal intestinal permeability

Abnormal intestinal permeability function has been recognized as a common feature of IBD. Increased permeability has been clearly demonstrated in a significant proportion of CD patients by down-regulation of tight junction proteins (for review see [188]). Abnormally elevated permeability has also been demonstrated in healthy first-degree relatives of CD patients [64]. There is evidence from animal models of IBD that abnormal permeability may precede the development of the disease [188]. Genetic susceptibility loci containing genes that are involved in barrier function have been implicated in both UC and CD, although these are not overlapping loci between the two forms [6,63]. It is possible that increased intestinal permeability may be an early step in the pathogenesis. This may lead to excessive antigen

exposure to the mucosal immune system, which results in an exaggerated immune response in a genetically predisposed individual [188]. Increased permeability in patients with clinical remission predicts a high risk of relapse [210]

1.3.4 Immune dysregulation

Despite tight and highly evolved regulation, dysfunctions of the immune system can arise, leading to hyper-inflammatory conditions and autoimmune disease. The inflammation is believed to be the result of several events and factors as numbered below [15]:

I. The epithelial barrier in IBD patients has a lowered resistance and increased permeability that precedes the clinical onset of the disease.

II. The innate immune mechanisms of the epithelial layer are disturbed, with an altered pattern of TLR expression. For instance, TLR4 is highly upregulated in addition to higher expression of NOD2 in epithelial cells.

III. There is a disturbed antigen presentation. Commensals are presented as pathogens, and DCs lack the regulatory capacity they normally have.

IV. IECs, for instance, become antigen presenting cells and activate T cells instead of inducing anergy.

V. There is a disturbed clearance of auto-or over-reactive T cells. Activated T cells do not undergo apoptosis.

VI. Both the innate and adaptive immune system is involved in the regulation of immune response, but T cells have been most tightly correlated with disease pathogenesis. There is a disturbed balance of regulatory and effector T cells. Effector T cells dominate over Tregs during active disease (for review, see [15,92,113]).

1.3.4.1 T cell mediated immune response in CD

CD has traditionally been considered a Th1 mediated disease, characterized by increased production of IFNG, TNF and IL12 and the Th1 related regulators and transcription factors [181]. After the discovery of IL17 as a potent mediator of inflammatory response, for instance by enhancing the effect of TNF [93], Fujino and colleagues published a report in 2003 showing that IL17 expression was increased in the mucosa of CD as well as UC patients [66]. Since then there have been several reports implementing both Th17 and Th1 in the pathogenesis of CD [67,97,112]. Strober and Fuss suggest that Th1 and Th17 responses are in a state of coexistence in CD, and that the balance between them will depend on the state of

disease activity. Th1 activity will tend to dominate in the most intense phase of inflammation [182].

1.3.4.2 T cell mediated immune response in UC

The immune response in UC has been considered less specific, and was initially characterized as an atypical Th2 response mediated by NKT cells producing IL13 and IL5 [68]. TNF is elevated in the mucosa of active UC and correlates to the grade of inflammation [137]. IL17 has also been found elevated in UC mucosa in several reports, thus implicating Th17 cells in the pathogenesis of UC [66,97,149]. Genetic signatures of UC and CD overlap to some degree, including genes involved in the regulation and function of T cells [6,10]. An enhanced accumulation of Treg cells have been demonstrated in inflamed mucosa of both forms of IBD [203]. Overall, there is an imbalance between regulatory and effector T cells in the pathogenesis of UC (for review, see [138]).

1.3.4.3 IBD and associated cytokines

The cytokine responses characterizing CD and UC are the key pathophysiologic elements that govern the initiation, development and the resolution of the inflammation. Some cytokines are strongly associated to the severity of the disease, and could be future candidate biomarkers of the clinical course and in therapeutic decision making (for review, see [61]).

Pro-inflammatory cytokines; TNF, IFNG, IL12, IL17A, IL23, IL6, IL21, IL33

TNF is produced in the intestine of mice with experimental inflammation [129], and excessive production is also seen in the inflamed mucosa of IBD patients [110,137]. TNF triggers multiple inflammatory pathways (described in section 1.2.4.1). TNF has been implicated in both forms of IBD [92,137], consistent with anti-TNF treatment being effective in both forms. It has been shown to correlate positively with the degree of inflammation in IBD mucosa [137]. **IFNG** is produced in increased amounts by LP T cells in inflamed CD, and is, in addition to other Th1 related cytokines, still considered the main driver of inflammation in CD (for review see [182]). **IL12** production is increased in CD mucosa [67], and stimulation with IL12 resulted in increased IFNG in one report [149]. IL12 has also been found in increased amounts in inflamed mucosa of UC [101,130]. IL12 shares a subunit (p40) with IL23, both having a pro-inflammatory role in IBD and driving different pathways of inflammation (for review [92]). **IL17A**, the hallmark cytokine of the Th17 cell, is considered an important mediator of inflammation in IBD [66,97]. A protective role of IL17 in the

process of mucosal inflammation has been suggested, through inhibition of IFNG [92]. IL17A producing regulatory T cells have also been described [86]. In addition, a population of human T cells (Th1/Th17) that co-produce IL17 and IFNG in mucosa of CD patients was identified in one study [7]. Blockade of IL17A was ineffective in a randomised placebo-controlled trial, and higher rates of adverse events were observed, including fungal infections [88]. The authors suggested that IL17A has a role in maintaining immunologic homeostasis in the gut. **IL23** production in the intestine is increased during inflammation. This cytokine drives the T cell mediated colitis by stimulating production of IL17A in particular (for review see [5]). Blocking IL23 decreased production of several pro-inflammatory cytokines, including TNF and IFNG in one study [87]. Monoclonal anti-IL23 reversed active colitis in a T cell-mediated model in mice [58]. It has, however, been shown that homozygous carriers of risk-increasing IL23 receptor variants are more likely to respond to IFX [90]. **IL6** is increased in both forms of IBD and is thought to arise from non-T cells. IL6 targets T cells and macrophages and induces production of TNF, IFNG and IL1, thus stimulating an inflammatory state [92]. **IL21** is produced in excess in the inflamed intestine of IBD patients. It was described in one report as originating from activated CD4⁺ Th cells that co-expressed IFNG, mainly in CD patients [164]. IL21 is also involved in IL17 production, and blocking IL21 resulted in a significantly decreased level of IL17 in one study [149]. IL21 enhanced NK cell cytotoxic response, triggered pro-inflammatory cytokine production and Th17 cell differentiation in yet another study [106]. **IL33** has been shown to be upregulated in active UC [171,177].

Anti-inflammatory cytokines; IL10, TGFB

IL10 is an anti-inflammatory cytokine. It has been measured in increased levels in both forms of IBD [114](for review see [92,165]). Inactivation of IL10 in mice resulted in an increased production of IL12 and IFNG [146]. **TGFB** is another regulatory or inhibitory cytokine that has an unsettled role in IBD. It is involved both in Th17 differentiation (along with IL6 and IL21) and regulatory T cell development [20]. It is also suggested to have a role in facilitating repair, which could explain its elevated expression levels in IBD [157]. In addition, TGFB has been shown to reverse the increased tight junction permeability that is seen in IBD [188].

1.4 IBD therapy

Current guidelines on conventional IBD therapy from the European Crohn's and Colitis Organisation (ECCO) recommend a selective use of aminosalicylates, corticosteroids and immunosuppressant [50,193]. Further details on conventional therapy or surgical management are beyond the scope of this thesis. In the last decade the management of IBD has been changed by the advent of targeted therapy, also called biological therapy. These agents have been created with genetic technology and target the mediators of immune response in IBD. They are based on knowledge of the immunopathogenesis underlying the diseases and have greatly improved IBD management (for review see [24]).

1.4.1 Targeted therapy

The immune response in the mucosa of IBD results in an excessive production of inflammatory cytokines. Targeted therapies are engineered proteins (often antibodies) that inhibit or modulate the mucosal immune system. Therapeutic targets for biologic agents in IBD may be: antigen/antigen presentation, mediators of T cell activation, cytokines mediating inflammation, factors involved in adhesion and recruitment and injury/repair [24,29]. For instance, antibodies against CD40 ligand (important co-factor in T cell activation), CD4 and CD3 have been tested in IBD, with various results and side effects. Therapy with anti-inflammatory cytokine IL10 has been tried in CD patients with promising preliminary results, but not confirmed in the following RCTs. Adhesion molecule inhibitors like natalizumab (antibody against $\alpha 4$ integrin) and MLN-02 (antibody against $\alpha 4\beta 7$ integrin) have shown promising results in clinical trials, with some concerns about safety (for review, see [24]). Several inhibitors of pro-inflammatory cytokines have been developed, with the focus of this thesis being on anti-TNF therapy.

1.4.2 Anti-TNF therapy

The development of TNF antagonists was a result of identifying TNF as a main driver of inflammation in many autoimmune diseases. The first TNF blocker used in IBD was infliximab (IFX), followed by adalimumab (ADA) and certolizumab pegol. Data from clinical trials suggest that they have similar efficacy and adverse-event profiles; although no comparative studies have been performed. In Norway IFX and ADA have been approved for use in IBD treatment. IFX is a chimeric monoclonal antibody containing 25% mouse derived peptide and ADA is a fully human monoclonal IgG1 anti-TNF antibody [29]. Population-based and cohort-studies around the world have estimated that nearly 15% of IBD patients are

treated with anti-TNF therapy [42]. The following sections will give some insight into the mechanisms as well as the ongoing discussion regarding the use of these agents.

1.4.2.1 Mechanisms of action

As described in previous sections, TNF plays a central role in mucosal inflammation and is most likely a key mediator in the inflammatory cascade in IBD [11,182]. TNF antagonists bind to soluble and membrane forms of TNF and can neutralize their effects in immune-mediated inflammatory diseases [208]. The murine variable region of IFX can lead to production of anti-mouse antibodies potentially limiting therapeutic efficacy. In ADA only the hypervariable sequences are from mouse protein [208]. IFX and ADA bind TNF with high affinity and specificity, neutralizing it and preventing it from binding to its receptors, and also inducing cell lysis and apoptosis. Binding to membrane TNF can mediate apoptosis. These agents may also work by influencing intracellular signalling (reversed signalling) in other ways. They suppress cytokine production, thus decreasing the expression of several downstream pro-inflammatory cytokines and chemokines and inhibiting the following inflammatory effects [8,208]. In addition there have been reports on anti-TNF down-regulating expression levels of adhesion molecules on endothelial cells, thereby inhibiting T cell recruitment [46]. TNF antagonists also facilitate wound healing in the intestine by modulating fibroblast function [49]. Of interest was the report by Ricciardelli and colleagues [148], who demonstrated an increase in/restoration of FOXP3⁺ regulatory T cells in children with active CD treated with IFX to remission. Moreover, anti-TNF therapy has been shown to normalize permeability in both CD [184] and UC [190].

1.4.2.2 Therapeutic goals

Since its introduction over a decade ago anti-TNF therapy has been shown to effectively treat and heal inflamed mucosa of CD and UC. Over the past years, the major therapeutic goal has moved from symptom relief to mucosal healing (MH) for both CD and UC. MH is also a common end-point in clinical trials. The interest in MH was raised when it was demonstrated that anti-TNF treatment induced marked healing of intestinal lesions in patients with refractory luminal CD [142,197]. There has been accumulating evidence that MH changes the course of the disease, reduces complications, hospitalizations and need of surgery [141]. In the ACT 1 and 2 trials [154], the proportion of IFX treated patients with UC in clinical remission at week 30 was four-fold greater for patients with MH earlier on in the studies. The population-based study on IBD in south-eastern Norway (IBSEN study) showed that MH was

significantly associated with less inflammation after 5 years of follow-up [65]. This was supported by another study in 2010, which showed that complete MH was the only factor that predicted sustained steroid-free remission up to 4 years after therapy initiation [13]. MH is considered a more objective end point than clinical remission in clinical trials, but validated definitions of the term has been lacking for both UC and CD, although validated scoring systems exist for both diseases (for review, see [142]). This topic will be discussed later on in this thesis.

1.4.2.3 Duration of treatment; induction and maintenance therapy

According to the ECCO guidelines, anti-TNF therapy is indicated in steroid-refractory, steroid-dependent and/or immunomodulator-refractory IBD or in case of intolerance to these so called conventional therapies [50,193]. It is administered as an induction regime where response is assessed and then often continued as a scheduled maintenance therapy. As mentioned in the previous section, anti-TNF therapy has been associated with MH and favourable prognosis; however, it has also been associated with significant side-effects and opportunistic infections [144]. Rare but severe adverse events do occur [30], and patients receiving anti-TNF therapy should be selected carefully. There is currently no international consensus on when to stop ongoing anti-TNF therapy for patients in remission. The National Institute of Clinical Excellence (NICE; British guidelines) lists a number of recommendations regarding anti-TNF therapy of CD patients, and recommends a treatment course of one year after achieving clinical remission [127]. The position statement from the World Congress of Gastroenterology in 2010 says that there is no clear evidence and data is insufficient to make recommendations on duration of anti-TNF therapy. The same group also states that for patients in clinical remission for > 1 year, with normal CRP and MH, an appreciable proportion of patients will remain in remission the next year [42]. Concerns have been raised that biologics may lose efficacy when re-introduced after withdrawal [152]. However, recent reports have shown that response to re-treatment with anti-TNF agents is favourable after a period of drug withdrawal [108,180].

Table 2 Main randomized, placebo-controlled trials of anti-TNF induction and maintenance therapy in ulcerative colitis and luminal Crohn's disease

Study	IFX/ADA	Clinical remission after induction (placebo)	Clinical remission on maintenance (placebo)	Mucosal healing (placebo)
UC				
ACT I [154]	IFX ind/maint	39 % (15 %)	20 % (7 %)	62% wk 8 (34 %)
ACT II [154]	IFX ind/maint	34 % (6 %)	15 % (2 %)	60% wk 8 (31 %)
ULTRA1[145]	ADA ind	18.5 % (10 %)		47% wk 8 (42 %)
ULTRA2[160]	ADA maint		17.5 % (8.5 %)	25% wk 52 (15%)
CD				
Targan [186]/Rutgeerts [150]	IFX ind/maint	33% (4%)	53 % (20 %)	
ACCENT I [74]	IFX maint	58 % response	28 % % (11 %)	
CLASSIC I [75]/II [159]	ADA ind/maint	36 % (12 %)	79 % (44 %)	
CHARM [35]	ADA ind/maint	58 %	36 % (12 %)	
EXTEND [155]	ADA ind/maint			27% wk10 (13 %) 24 % wk52 (0 %)

Table 2 shows the percentages of patients achieving clinical remission after induction and maintenance therapy of IFX or ADA, in the main randomized placebo-controlled studies performed since the introduction of anti-TNF therapy. Results for mucosal healing are listed if available. Results for placebo groups are in parentheses.

Abbreviations: ind=induction therapy, maint=maintenance therapy, IFX=infliximab, ADA=adalimumab

1.5 Predicting clinical outcome: biomarkers of relevance

Biomarkers in general can be defined as characteristics of a biological system that are measured in an objective manner and can be used as measures of the system of interest [109]. They are also defined by the National Institutes of Health (USA) as objectively measured indicators of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention [21]. Biomarker research is rapidly increasing and can potentially form the basis of personalized medicine, where the therapeutic approach is tailored to the individual patient. Surrogate markers are alternative markers that are less invasive or cheaper and substitute the clinical endpoint of interest [109]. In IBD there have been several studies on possible predictors of disease and therapeutic outcome. Possible predictors can be clinical and demographical parameters (for review see [99]). For instance, in CD, an initial need for steroids, the presence of perianal disease and age below 40 years were associated with disabling disease in one study [16]. Small bowel disease has been associated with progression towards more complicated disease or surgery in CD. Smoking is associated with more complicated disease in CD, but in UC it has been associated with a more benign disease course [99]. MH has been associated with improved disease outcome for both CD and UC [13,65,170]. Laboratory markers like ESR and CRP are traditional non-specific markers of inflammation. CRP has, for instance, been identified as a predictor of relapse in CD [36]. Serologic, genetic, stool and tissue markers are objective parameters that have been more or less evaluated as possible predictors of therapeutic and disease outcome (for review see [61,165]). Biomarkers can also be divided into prognostic and predictive markers, each providing information on either clinical outcome or therapeutic effect, respectively. Biomarkers are also possible indicators of MH.

As mentioned previously there are a number of known genetic susceptibility loci for both forms of IBD [6,63], and there have been reports linking genotype to clinical phenotype and therapeutic outcome. For instance, Dubinsky and colleagues found that 6 known IBD susceptibility loci were associated with primary non-response to anti-TNF therapy in pediatric IBD [53]. Genetic susceptibility loci have also been linked to a higher risk of colectomy [78]

Serological markers belong to the surrogate markers, i.e markers obtained by a non-invasive approach. The two best studied are pANCA and ASCA. pANCA+/ASCA serotype has been associated with a suboptimal response to IFX [59]. ASCA positivity was associated with a more aggressive disease course with a higher rate of complications in CD (structuring, penetrating disease) [123]. New serologic markers are also emerging (for review see [115]).

Fecal calprotectin, lactoferrin and S100AI2 are neutrophil derived proteins that can be measured in stool samples, all of them well correlated to grade of inflammation in IBD (for review see [115]). Fecal markers are considered good surrogate markers, and are utilized in clinical practice for early detection of relapse [115]

Mucosal markers originating from the mucosal immune response involved in IBD have been associated with disease activity and grade of inflammation in several reports (for review see [61]). *TNF* gene expression correlated to grade of inflammation in a report by our group in 2007 [137] and was significantly associated with outcome after infliximab therapy in a subsequent study [136]. In two reports by the Leuven group, mucosal gene expression associated with immune response and regulation in IBD were identified as accurate predictors of response to IFX [9,10].

1.6 Summary of introduction

Knowledge on the immunological mechanisms that mediate the inflammatory processes of IBD is gradually increasing, but the pathogenesis is far from fully resolved. Research in this field has established the foundation for targeting therapy, but this form of treatment is expensive, and not equally effective in all patients. When diagnosis is established we do not know for certain which factors that are involved in the development of complicated disease, which factors that determine therapeutic effect, and which factors that should be considered in choosing therapeutic strategy. Mediators of inflammation and their metabolites that correlate with disease activity could potentially be utilized in clinical practice as predictors of disease and therapeutic outcome. Data on predictive biomarkers for response to anti-TNF therapy are scarce, only a few reports exist on the prediction of long-term clinical outcome and risk of relapse. This brings me to the aims of this thesis.

2. Aims of the thesis/research questions

1. Do mucosal gene expression levels of T cell related cytokines differ between patients with CD and UC?
2. Is there a correlation between levels of expression of different cytokines and disease activity in IBD?
3. Can cytokine expression levels be utilized as predictors of outcome after anti-TNF therapy in IBD?
4. Is there an association between mucosal cytokine expression profile in healed mucosa and outcome after withdrawal of anti-TNF therapy?

3. Summary of results, study population and ethics

All papers are based on studies in the population of IBD in-and out-patients at the Department of medical gastroenterology at the University Hospital of North Norway between 2003 and 2011 (**Table 3**). Patients gave written, informed consent upon inclusion, and had the option of withdrawing from participation after inclusion. The study was approved by the regional board of research ethics. Establishment of a biobank was approved. Tables with demographical and clinical characteristics are included in the respective papers.

Table 3 Study material and design in paper I-IV

	Patients screened	Design	Period	Included	Lost to follow-up/excluded
Paper I	257 UC 75 CD	Cross-sectional	2003-2008	79 untreated UC 32 untreated CD 23 controls	N.A
Paper II	90 UC patients starting IFX	Prospective	2003-2011	74 UC patients 19 Controls	16
Paper III	77 CD eligible for ADA	Prospective	2008-2011	77 CD patients 17 controls	7
Paper IV	84 CD patients starting anti-TNF	Prospective	2008-2012	37 CD patients with MH and anti-TNF discontinuation 17 controls	4

Nearly all IBD patients are examined by endoscopy at some point. In our clinic biopsies are taken routinely for real-time quantitative PCR (RT-qPCR, described and discussed in section 4.1.2.2) for measurement of mucosal TNF expression. Refusal to participate in the study has not been an issue since participation does not involve more extensive follow-up than normal. Available biopsies for RT-qPCR analyses are, in addition to being without any treatment for the past 3 months, the main determinant for inclusion in **paper I**. Lack of post-treatment endoscopy or biopsy determine number lost to follow-up in **paper II**. ADA is currently first choice for patients with luminal CD and no patient eligible for ADA refused participation in the prospective study in **paper III**, the flow-chart in the paper gives a more detailed description. In **paper IV** patients with MH from the cohort of CD patients included in paper III were included, in addition to patients treated to MH by IFX. Patients who did not discontinue anti-TNF therapy upon documentation of MH were excluded. Inclusion of IBD patients and controls is commented and discussed under selection bias in section 4.1.2.1.

3.1 Paper I

“Th1 and Th17 interactions in untreated inflamed mucosa of inflammatory bowel disease and their potential to mediate the inflammation”

Background & Aim: Crohn’s disease (CD) and ulcerative colitis (UC) have been associated with a T helper1 (Th1) and a Th2 cytokine profile, respectively. Recently, a Th17 lineage has been introduced, but their role in the inflammation of CD and UC is not fully understood. The aim of this study was to characterize the cytokines directing the Th17 cells and their interactions with Th1 cells in the mucosa of untreated patients with CD and UC. **Method:** Seventy-nine patients with untreated UC, thirty-two patients with untreated CD and 23 controls with no signs of colon disease were included in the study. Clinical indices for Ulcerative colitis (UCDAI) and Crohn’s disease (CDAI) were assessed. Biopsies for measurements of interleukin-17A (*IL17A*), *IL23*, *IL6*, transforming growth factor-beta (*TGFB*), interferon-gamma (*IFNG*) mRNA levels as well as immunohistochemical (IHC) analyses were performed. **Results:** The gene expression for all cytokines in UC and for all cytokines except for *TGFB* in CD were significantly increased compared with the controls. The IHC analysis showed significantly increased number of *IL17A* positive cells in lamina propria and epithelium of both UC and CD compared to controls. The levels of *IL17A* and *IL23* mRNA were significantly higher in UC than in CD while the levels of *IL6* were significantly higher in CD compared with UC. The levels of *IL17A*, *IL6* and *IL23* mRNA were associated with the disease activity score in both UC and CD. *IFNG* expression was associated with the disease activity in UC, but did not reach significant level in CD. **Conclusion:** Increased levels of *IL17A* and *IL23* were found in both UC and CD compared to controls. Association to the grade of inflammation and clinical activity was also observed. *IL17A* and *IL23* were significantly higher in UC than in CD. Th1 and Th17 cytokines seem to act synergistically in inflammatory bowel disease (IBD) with no apparent polarization between UC and CD.

3.2 Paper II

“Mucosal cytokine gene expression profiles as biomarkers of response to infliximab in ulcerative colitis”

Objective: Mucosal cytokine profile determines T cell differentiation and may play an important role in the clinical course of inflammatory bowel disease (IBD). Cytokines from different T helper (Th) cell subsets are elevated in inflamed mucosa of patients with ulcerative colitis (UC), contributing to the inflammation. The aim of this study was to determine the

predictive value of pre-treatment mucosal cytokine profile in response to therapy with the anti-TNF agent infliximab (IFX). **Material and methods:** The expression of Th1, Th17, Th2 and T-regulatory (Treg) related cytokines was quantified by real-time PCR in mucosal biopsies from 74 UC patients before initiation of IFX induction therapy. Clinical and endoscopic effects were assessed after 3 infusions. Remission was defined as Ulcerative Colitis Disease Activity Index (UCDAI) below 3. **Results:** Higher gene expression levels of interleukin-17A (*IL17A*) and interferon-gamma (*IFNG*) were significantly associated with remission after 3 IFX infusions (OR=5.4, p=0.013 and OR=5.5, p=0.011, respectively). *IL17A* and *IFNG* mRNA expression showed positive correlation. Th2 and Treg related mediators were not significantly associated with clinical outcome, but were expressed at higher levels in UC patients compared to the controls. Immunohistochemistry (IHC) confirmed the presence of cells co-expressing IL17A and IFNG. **Conclusions:** High expression of Th1 and Th17 related cytokines in the mucosa of UC patients can potentially predict a favourable outcome of IFX induction therapy. Th2 and Treg related mediators do not appear useful as predictive markers.

3.3 Paper III

“The effect of adalimumab for induction of endoscopic healing and normalization of mucosal cytokine gene expression in Crohn’s disease”

Objective: To investigate the effects of adalimumab on the induction of complete endoscopic healing and normalization of mucosal cytokine gene expression in patients with active Crohn’s disease. **Material and methods:** A prospective, single-centre study including 77 patients. All were examined by endoscopy before initiation of adalimumab induction therapy with a minimum of 6 adalimumab injections. Patients were treated until documentation of complete endoscopic healing. Biopsies for measurements of mRNA expression levels of interleukin(IL)-17A (*IL17A*), *IL23*, interferon-gamma (*IFNG*), tumour necrosis factor-alpha (*TNF*), *IL10* and Forkhead Box P3 (*FOXP3*), as well as for immunohistochemistry (IHC) were sampled at pre-and post-treatment endoscopy, and from 17 control patients. **Results:** Complete endoscopic healing was achieved in 27.3% after 10 weeks of treatment, documented by endoscopy at week 12. Cumulative endoscopic healing after 52 weeks was 44.2%. Complete endoscopic healing led to a significant reduction in mRNA expression levels for all cytokines except *IL10*. Elevated expression of *TNF* and *IL-17A* persisted in 52% and 76%, respectively, of patients with complete endoscopic remission. Pre-treatment cytokine gene expression levels did not predict response to adalimumab therapy.

Conclusions: Adalimumab induces accumulated complete endoscopic healing in 44% of patients after 52 weeks of therapy. Normalization of mucosal gene expression of cytokines does not occur in all patients with endoscopy-verified healed mucosa. Inclusion of normalized mucosal cytokine expression into the concept of mucosal healing could have an impact on long-term clinical outcome.

3.4 Paper IV

“Normalization of mucosal cytokine gene expression levels predicts long-term remission after discontinuation of anti-TNF therapy in Crohn’s disease”

Objective: To investigate mucosal cytokine gene expression levels in healed mucosa after anti-tumour necrosis factor (TNF) therapy in patients with Crohn’s disease (CD) as possible risk factors for relapse after discontinuation of therapy. **Design:** Thirty-seven CD patients were treated with anti-TNF agents until complete mucosal healing was documented by endoscopy; after which anti-TNF treatment was discontinued. Levels of mRNA expression levels of interleukin-17A (*IL17A*), *IL23*, interferon-gamma (*IFNG*), *TNF*, *IL10* and Forkhead Box P3 (*FOXP3*) were measured in mucosal biopsies and analyzed as possible risk factors of relapse. Mucosal cytokine transcript levels from patients without CD served as controls.

Results: Patients were followed after discontinuation of therapy until relapse. Median time to relapse was 26 weeks, and 7 (26%) of 27 evaluated patients were still in remission at week 52. In patients with normalized *TNF* or *IL17A* mucosal gene expressions 63% and 67% were still in remission after 52 weeks, respectively. Expression levels of *TNF*, *IL17A* and *FOXP3* were significantly higher in patients who relapsed before 26 weeks than in those who did not relapse, and also higher in patients with relapse before week 52 versus non-relapsers. Elevated expression levels of *TNF* and *IL17A* in healed mucosa significantly increased the risk of relapse (HR=3.4, p=0.03 and HR=4.1, p=0.008, respectively). **Conclusions:** Normalization of mucosal gene expression of cytokines after anti-TNF therapy does not occur in all patients with completely healed mucosa as judged by endoscopy. Normalization of *TNF* and/or *IL17A* expression predicts long-term remission.

4. General discussion

Detailed application of the methods, presentation of results and discussions can be found in the respective papers I-IV. In this general discussion I will first give a combined theoretic overview and discussion of the methods. Following this is a discussion of the main results.

4.1 Methodological considerations

4.1.1 Study design

An overview of the population and design in the respective papers has been presented above (table 3). The cross-sectional design in paper I restrict the possibilities of concluding as far as the direction of the associations detected. In paper II-IV the study designs were prospective. With this design it is possible to conclude with regard to the predictive impact of the various cytokine profiles on the selected outcomes.

4.1.2 Internal validity and bias

Internal validity refers to which extent it is possible to draw conclusions concerning the study population. The term is classified into selection bias, information bias and confounding [17].

4.1.2.1 Selection bias

Selection bias may arise when the subjects included in a study differ from the source population. Patients in our studies were recruited consecutively from a specialist department and went through similar examination, treatment and follow-up as any IBD-patient in our department, and therefore selection bias was considered not be an important issue. The study population for paper I were patients with both inactive and active IBD who had an endoscopic examination as part of their regular follow-up or due to a flare. Patients in papers II-IV were followed just as usual during anti-TNF therapy. The control patients had normal colonoscopy and histological examination, but had been referred and examined based on symptoms from the GI tract. This could potentially be a source of selection bias, as an altered immune intestinal immune response in the control patients can not be completely excluded. The control group reflected the fold increase in cytokine mRNA expression in IBD patients (untreated in paper I, before treatment in paper II and III, and after treatment in paper III and IV). It is possible that the potential selection bias of the control group would in fact decrease the difference in expression level between IBD patients and controls, but this question will remain unanswered. Patients with intestinal adenocarcinoma and dysplasia were not included

as control patients since immunological changes have been demonstrated in tissue adjacent to the tumor [39]. IBS patients were not used as controls, although some of the symptoms presented by subjects referred to endoscopy could be explained by IBS. Some IBS symptoms can be seen as a part of normal bowel function. Patients with IBS have normal endoscopic and histological findings; however, an altered immune response is thought to play a role in development of IBS [111]. Ideally, controls should be recruited from the healthy background population, but this is difficult if not impossible to achieve. The different number of controls in the different papers is partly due to limited amount of RNA available for RT-qPCR analyses, which made it necessary to add new controls to the material.

4.1.2.2 Information bias

Information bias result from systematic errors in the information obtained about the study participants and the main source of bias in this study. Information bias will be discussed in the following in relation to the description of RT-qPCR, IHC, assessment of disease activity and endpoint registry.

Quantitative real-time reverse transcription-polymerase chain reaction

The procedure for performing PCR was first introduced by Kerry Mullis in 1983 for which he won the Nobel Prize in 1993. The method has had a great impact on biological research since [52]. Quantification of RNA transcripts became available with the advent of RT-qPCR. RT-qPCR enables rapid identification, screening, classification and monitoring of RNA targets and is established as the current method of choice for the accurate detection of RNA. With the ongoing sequencing of the human genome and identification of disease-associated polymorphisms, RT-qPCR technology has a wide range of translational applications (for review see [125]. There are, however, significant biological as well as technical limitations that make the use of this sensitive method challenging. It is essential that the RT-qPCR assays target appropriate biomarkers, use suitable and validated assays and are properly analyzed [27,28]. The following sections will present the method in general and discuss the potential limitations.

Method description

RT-qPCR is used for detection and measurement of products generated during each cycle of the PCR process which are directly proportionate to the amount of template prior to the start of the PCR process [69]. RT-qPCR is a combination of 3 steps: 1. Reverse transcriptase (RT)-

dependent conversion of RNA into cDNA, 2. Amplification of cDNA using PCR, and 3. Detection and quantification of amplification products in real time [52,131]. During the PCR procedure, changes in temperature are used to control the activity of the polymerase and binding of primers that are used to copy the gene of interest. Fluorescent reporter dyes are used to combine the amplification and detection steps of the PCR reaction in a single tube format. The assay relies on measuring the increase in fluorescent signal, which is proportional to the amount of DNA produced in each PCR cycle. Individual reactions are characterized by the PCR cycle at which fluorescence first rises above the threshold of background noise, also known as the threshold cycle (C_T). The more template present initially in the sample the lower the C_T , since the threshold is reached sooner [131]. The C_T values of different samples are used to calculate the relative abundance of template for each sample.

For the detection of RNA or DNA targets our laboratory used TaqMan technology, which is a probe-based chemistry developed to increase the specificity of the assay. The probe consists of a “middle” primer conjugated with a reporter dye on the 5' end and a quencher dye on the 3' end [52]. TaqMan probes anneal to a target DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore (reporter dye) from the close proximity to the quencher dye, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Fluorescence detected in the real-time PCR thermal cycler is directly proportional to the amount of DNA template present in the PCR [52].

Assay design: primers and probes

The reliability of the assay depends on an optimal assay design. Assays can be designed using available design software, it can be bought by specialized companies, or published and validated assays are available in public databases. The assays used in paper I-IV were designed using Primer Express, except for IFNG which was bought from Applied Biosystems. Primer sequences were listed in paper I-II for those not reported previously by our group [70]. Developing an ideal assay includes choosing primers that will result in an amplification product that is specific to mRNA and not amplification of genomic DNA, which could potentially contaminate the RNA. Placement across an exon splicing point ensures that only processed mRNA is detected [69]. Keeping up to date with the continually increasing number of annotated single-nucleotide polymorphisms (SNPs) is important when designing RT-qPCR

assays, particularly when working with samples that may have different or unknown genetic backgrounds.

RNA quality

RT-qPCR reproducibility is affected by RNA quality. Sampling of biopsies, storage and extraction process are critical steps, and have been evaluated in our laboratory [38]. An RNA quality scoring system, called RNA integrity number (RIN), has been developed [60]; a RIN higher than 5 indicates good quality and higher than 8 as perfect total RNA. We used *RNAlater* (Ambion Inc), an aqueous, non-toxic tissue storage reagent that rapidly permeates tissue to stabilize and protect cellular RNA in situ in unfrozen specimens, without jeopardizing the quality or quantity of RNA. *RNAlater* eliminates the need to immediately process specimens or freeze them in liquid nitrogen for later processing. In a validation study by Ellis et al [57] it was demonstrated that *RNAlater* and liquid nitrogen preserved RNA quality equally. Tissue placed in *RNAlater* can be stored at 25 ° for 1 week and at 4° for up to 1 month without evidence of tissue degradation, according to the manufacturer's protocol. Procedures in our laboratory were evaluated in 2006 [38] and it was concluded that *RNAlater* exhibited a high preservation effect against RNA degradation even after 8 days storage at room temperature, with RIN-values above 8.

RNA isolation was performed using two different methods, the Trizol (paper I and II) and the Promega method (paper II-IV). In paper II, RNA was extracted from biopsies by both methods. Using different methods for isolating RNA could potentially result in differences in RNA purity and yield, and differences in amount of contaminating DNA. Potential systematic differences between isolation methods were assessed by running an ANOVA on beta-actin (*ACTB*, house-keeping gene) expression; no differences were detected across extraction methods.

Reverse transcription

The conversion of mRNA to cDNA is a sensitive step in the quantification process. RT-qPCR gene expression measurements are only comparable when the same priming strategy and reaction conditions are used in all experiments and the samples contain the same amount of RNA. Experimental accuracy is improved by running samples in duplicates [178]. Stahlberg and colleagues reported up to 100-fold variance in reverse transcription yields between different choices of reverse transcriptases [179] Reverse transcription were performed using

iScript in all our experiments. We used the same protocol and reagents in all the experiments to minimize the effect of this potential source of technical variability.

Normalisation

Data normalisation is an indispensable component of RT-qPCR analysis, essential for comparison of RT-qPCR measurements between different samples. Normalisation controls for the total mass of RNA analyzed, and ideally, corrects for any biological and technical variability [52]. Sample amount should ideally be approximately the same size (we used two biopsies per sample). There are, however, large histological differences in intestinal biopsies, and nucleic acid extraction might be very different. Total RNA amount used for reverse transcription should be the same; total RNA concentration was measured in our experiments at 260nm with U-1500 UV/Vis spectrophotometer (paper I and II), and NanoVue spectrophotometer (paper III and IV). The use of internal reference genes is the most common method for normalisation. This strategy targets RNAs that are assumed to be universally expressed, and do not differ between experimental and control groups or under different circumstances. The identification of a valid reference for data normalization to achieve accurate, reproducible, and biologically relevant mRNA quantification is also a problem; especially when it comes to comparing gene expression profiles using in vivo biopsies from different individuals [27]. Accurate comparison between samples cannot be done without appropriate normalisation. There have, however, been reports that demonstrate that the classic reference genes are subject to a considerable amount of variation and might be unsuitable for the purpose of normalisation [194]. Reference genes should be evaluated in each experiment. We used beta-actin (*ACTB*) in all the experiments, and to investigate the stability of *ACTB* as house-keeping gene, an analysis of variance (ANCOVA) was performed including all clinical parameters (age, gender, disease/treatment/control, healing/non-healing, etc). No significant effects on expression of *ACTB* were found, and it was considered to be a suitable house-keeping gene for our experiments.

Heterogeneity of material

In our studies whole tissue biopsies were sampled and extracted for RNA. Thus, the RT-qPCR results are an average of the mRNA expression of cytokines in different cells of the intestine. We used immunohistochemistry (IHC) to confirm the presence of immune cells positive for relevant cytokines. Our laboratory has not yet established a method for sorted cell

analyses on intestinal biopsies, but there have been other studies on sorted intestinal T cells [97], confirming some of the results reported in paper I.

Reproducibility

All samples were analyzed in duplicates, and it has been demonstrated previously that variability is low [38]. All runs included two negative controls, to assess the possibility of contamination. Two positive controls with known C_T values were added in each run, to assess reproducibility. Reproducibility of gene expression between adjacent mucosal biopsies has been assessed by Wu and colleagues, who analyzed gene expression patterns in mucosal samples taken 10 cm apart in one intestinal segment of a CD patient. They concluded that the gene expression pattern of a single endoscopic pinch biopsy was a highly reproducible reflection of the gene expression in that given diseased segment of the intestine [209].

Relative quantification and the comparative C_T method

The quantitative endpoint for RT-qPCR is the threshold cycle (C_T). The C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold, placed in the exponential phase of amplification. The numerical value of the C_T is inversely related to the amount of amplicon in the reaction [169]. There are several ways to report RT-qPCR data, both as absolute and relative expression levels. Absolute expression provides the exact copy number following transformation of the data via a standard curve [26]. In relative quantification the data is presented relative to another gene. The comparative C_T method is one way of presenting RT-qPCR data, also known as the $2^{-\Delta\Delta C_T}$ method [169]. The comparative C_T method makes the assumptions that the efficiency of the PCR is close to one and that the efficiency of target gene is similar to the internal control gene [169]. We used relative quantification and the comparative C_T method in all 4 papers, which is a widely used method to present real-time PCR data, especially for research purposes.

Interpretation of mRNA versus protein expression

RT-qPCR data only constitute a snapshot of information regarding the quantity of a given transcript in a cell or tissue. Any assessment of the biological consequences of variable mRNA levels should ideally include additional information regarding protein levels and protein activity [131]. Proteins are the direct executors of life processes and probably reflect gene function more directly than mRNA. A key assumption in studying mRNA expression is that it is informative in the prediction of protein expression. Guo and colleagues reported

significant correlations between mRNA and protein expression. Protein was measured by mass spectrometry and some variation in the strength of correlation was reported, both for groups of proteins and within the individual [72]. Lack of correlation between mRNA and protein can be explained by biological processes like transcriptional and post-transcriptional splicing, translational modification and regulation, protein complex formation, and also mRNA and protein degradation rates. Functional genomics approaches should be developed, and the lack of this is a possible shortcoming in our studies.

Immunohistochemistry

IHC is a qualitative or semi-quantitative method for localizing specific antigens in tissues or cells based on antigen-antibody recognition, in other words a method for visualising cells or tissue that contain or produce the protein of interest [43]. IHC was applied in paper I, II and III to verify the expression of proteins/cytokines in mucosal samples. There is a thorough description of the procedure in each paper. IHC has traditionally had a role in demonstrating cellular proteins and identifying the cellular source. There are almost an infinite number of antibodies to specific proteins available [103]. There are a number of factors to take into consideration as far as reproducibility is concerned. There is potential bias both in the technical aspect of the method and in the interpretation of the results. Pre-analytical factors are primarily related to specimen handling, fixation, paraffin embedding and sectioning. Analytical factors relate to method of antigen retrieval, staining method, reagent validation, and use of controls/references. Post-analytical factors relate to interpretation of results and reporting [187].

Sample preparation is the least standardized phase of the staining process. Formalin fixation is not standardized as far as concentration, buffers etc, and varies from laboratory to laboratory. This will influence the outcome of immunostaining, reproducibility and generalizability [102,187]. There is a minimum time recommendation for formalin fixation, as well as a maximum time. It is likely that certain antigens have optimal fixation duration [102]. In our laboratory biopsies were directly immersed in formalin without delay, and duration of formalin fixation was less than 24 hours. Overall, the sample preparation steps were kept as consistent as possible, involving the same technicians and IHC investigator throughout the duration of the project.

Antigen retrieval is subject to a great variation in performance, which may affect the intensity of stain achieved or number of cells perceived as positive [187]. The increasing range of available antibodies can be a challenge as far as sensitivity and specificity is

concerned. There are a number of antibodies that targets the same antigen, but different epitopes, which may affect sensitivity [103]. In our procedure, testing of several antibodies was performed, and a selection made based on the sensitivity.

Interpretation of immunostains should always be made in the context of the known localization or distribution pattern of the targeted antigen. By semi-quantitative technique the density of positive cells can be counted and graded. In general the density of positive cells will reflect the amount of target protein. This quantitation is subject a significant amount of intraobserver variability [102]. When controls and test material are subjected to the same pre-analytical and analytical variables, they can be considered comparable. This is difficult to achieve, and at best the quantifications of stains are approximations.

Appropriate positive and negative tissues and reagent controls are the highest form of quality control of the immunohistologic assay. The negative control slides were performed routinely: primary antibodies were substituted with matched control antibodies.

Double staining IHC is a method for visualizing co-localized proteins in the same cell, and was used in paper II to see if IL17A and IFNG were expressed in the same cells, in paper III parallel sections were used to visualise co-localization of IL17A and TNF. With double staining two different proteins co-localized in the same cell are visualised by two different colors. However, cross-reaction of antibodies can give false positive results. In addition, the colors must contrast each other to avoid being mixed. Use of parallel sections will avoid the cross-reaction between two secondary antibodies.

IHC was used in order to confirm the expression of immune mediators on protein level. Ideally a method for quantification of protein should be applied, but our assays have not been sensitive enough (western blot). Mass spectrometry is an alternative and a highly sensitive method for identifying and measuring protein. Parallel analysis of the genome and the proteome could help discover post-translational modifications of gene transcripts.

Assessment of disease activity scores and end-point registry

UCDAI and CDAI

Disease activity for ulcerative colitis is often determined by measuring symptoms and signs. In this study it was assessed by the Ulcerative Colitis Disease Activity Index (UCDAI), which is a composite clinical and endoscopic disease activity index. Sutherland et al reported the results of a placebo-controlled 5-ASA trial in 1987 and described an instrument to measure disease activity called the Sutherland index, Disease Activity Index (DAI) or UCDAI [185].

Four variables determine a score ranging from 0-12: stool frequency, rectal bleeding, mucosal appearance and physician's rating of disease activity. The UCDAI score remains to be validated, but is commonly used in clinical trials (for review [40]). However, some of the symptoms listed in the index can be concurrent irritable bowel syndrome (IBS) related and not an indication of IBD activity. Use of the endoscopic sub-score will to some degree limit the effect of this source of information bias.

Disease activity in CD was assessed by the Crohn's Disease Activity Index (CDAI). This index consists of 8 clinical and symptom-based variables, and results in a score ranging from 0-600 points. Remission is defined by CDAI<150, mild activity 150-219, moderately active 220-450, and severe>150. The index was developed and validated in 1976 [19], and is still the most frequently used clinical index in trials (for review [158]). However, a considerable degree of inter-rater disagreement in recording and calculating the score has been reported [161]. The calculation is based partly on a 7-day symptom diary maintained by the patient, and partly on objective clinical parameters. The score was calculated by the principal investigators in paper I, and mainly by trained nurses and doctors in the out-patient IBD clinic in paper II-IV. The CDAI has been criticized for correlating poorly with endoscopic activity [31]. The lack of endoscopic grading of disease activity in CD is an obvious limitation in paper I, as far as interpreting the correlations observed between cytokine expression level and disease activity. In paper III the pre-treatment evaluation and inclusion criteria also included an endoscopic assessment, and patients had documented intestinal ulcerations, although no endoscopic grading score was applied. Endoscopic activity may be reliably scored with one of the validated endoscopic activity scores, but these are rather complicated and their use often restricted to clinical trials [47]. Implementation of an endoscopic scoring system into the routine follow-up endoscopies at our department has not been done yet.

Misclassification error can result from a study subject being placed in the wrong category [17]. Functional symptoms in both scoring systems and inter-rater disagreement are potential sources of misclassification of clinical status for both UC and CD. An endoscopic assessment will limit the possibility of misclassification. Further details on clinical classification and end-point registry in respective papers are provided below.

Endoscopic remission (paper II-IV)

In paper II, we included UC patients before initiation of infliximab therapy. The endpoint was remission, defined by UCDAI < 3 and endoscopic subscore of 0-1. This is a widely accepted definition, but the index has not been formally validated and inter-observer variation has to be taken into consideration. Over the past years, mucosal healing has emerged as a major therapeutic goal in clinical trials in IBD. Endoscopic remission or MH in UC has been defined in large clinical trials as endoscopic subscore of 0-1, and is a generally accepted definition [154]. There have, however, been concerns about the lack of a validated definition of MH. Mucosal friability is, for instance, allowed by some but not others in the definition of healed mucosa [47].

For CD, the Crohn's Disease Endoscopic Index of severity (CDEIS) has been developed. This score has been used in clinical trials both as primary and secondary endpoint, but it lacks a defined cut-off for remission [41,155]. Although several scoring systems for endoscopic appearance in CD are available and have been validated [48], the term mucosal or endoscopic healing lacks a generally accepted definition [47]. Both complete absence of signs of inflammation (redness) and absence of ulcerations have been used to define MH [96,155]. We defined MH in CD as complete healing with absence of any signs of ongoing inflammation in the ileocolonic mucosa. Since patients were sometimes examined by different doctors, applying this strict definition most likely reduced the potential for inter-observer variation. The concept of transmural healing, which is eradication of inflammation in all the layers of the intestinal wall, has not been used as end-point in any clinical trial, and lacks a reliable definition. The absence of endoscopic mucosal lesions does not exclude the possibility of sub-mucosal inflammation [47], and this increases the risk of misclassification.

Relapse (paper IV)

Time to relapse was the defined endpoint in paper IV, which was a prospective study performed in a cohort of CD patients after discontinuation of anti-TNF therapy. Clinical relapse in CD has been defined as CDAI > 150 points and an increase in the CDAI of 60 or 100 points. We defined relapse as a CDAI increase of > 70 points from the time of discontinuation, as recommended by Sandborn et al [158] and also used in other studies [108], and/or endoscopic findings qualifying for re-treatment with anti-TNF agent or systemic steroids, declared by the gastroenterologist performing the endoscopic examination or patient consultation. No endoscopic scoring system was applied for the assessment of endoscopic relapse. For practical reasons patients were generally not examined by endoscopy when a

flare was suspected, but evaluated by clinical assessment and calculation of CDAI, in addition to monitoring fecal calprotectin. Endoscopy was performed if there was any doubt. The only existing recommendation for the use of an established endoscopic scoring system in trials is for the assessment of post-operative recurrence, which has been defined using the Rutgeerts score [153,158].

4.1.2.3 Confounding

Confounding occurs when the observed association between variables is in fact an association between a different variable and the endpoint of interest. Confounding variables are related both to the exposure and the outcome [17]. Smoking status could, theoretically, be related both to mucosal inflammatory activity and response to therapy. Age and gender are common confounders, and are usually adjusted for in statistical analyses, which were also done in the relevant analyses in our studies. Clinical and demographical analyses including assessment of statistical differences between sub-groups were performed in all papers. Loss of power prevented us from extensive multivariate analyses and residual confounding and effect-modification cannot be excluded.

4.1.3 External validity

External validity or generalisability describes the extent to which research findings can be applied to other settings than the setting in which it was performed, or in this case generalising our findings to other circumstances or other IBD patients. Genetic differences between our cohort of IBD patients and other populations could potentially influence the generalisability of our results.

4.1.4 Statistical considerations

Statistical methods are described in detail in all papers. In general, all tests were 2-sided, significance level set to 0.05, and frequency distribution of ΔC_T values were assessed by visual inspection of histograms, tests of normality and residual plots. In cases where extreme values could be identified, analyses were re-run without these observations in order to evaluate if the outlier affected the conclusions. For the statistics in paper II, logistic regression was applied; results were reported as odds of remission and were at significant level. It should be taken into consideration that small to moderate size studies employing logistic regression as analytical tool can risk overestimating the effect. The phenomenon of small studies reporting larger effects than large studies is called the small studies effect [128]. In general,

when dealing with small study samples there is a limit to how many variables that can be included in a multivariate analysis (1/10 events). In small samples statistically significant effect might not occur when adjusting for possible confounders, this is a potential source of type II error. The study sample sizes in our studies were to a large extent based on what was practically possible in terms of how many patients we were able to recruit.

Due to lack of predefined cut-offs for normalization of cytokine gene expression or different levels of elevation, an arbitrary selection of cut-offs for high/medium/low mRNA expression and normalization, respectively, were employed in paper II and III-IV. An arbitrary unit (AU) is a relative unit of measurement to show the ratio of amount of substance to a predetermined reference measurement. The reference measurement is typically defined by the local laboratories or dependent on individual measurement apparatus. The unit only serves as a placeholder unit to compare multiple measurements performed in similar environment [135]. These selected cut-offs would have to be validated in follow-up studies before implementation into clinical practice.

4.2 Discussion of main results

Detailed discussions of the main results can be found in the respective papers I-IV. In the following sections the classification of IBD in the context of immunophenotype and our contribution to this topic will be discussed. The general aspects of immune mediators as biomarkers, and finally discussions on the concept of MH and the utility of immune markers in therapeutic decision making will be discussed in relation to our findings.

4.2.1 Immunophenotype and disease classification

Do mucosal gene expression levels of T cell related cytokines differ between patients with CD and UC? Over the years efforts have been made to classify CD and UC on the basis of T cell lineages that characterize the two diseases. In an attempt to place them within the Th1/Th2 spectrum, CD and UC have been viewed as a Th1 and an atypical Th2 driven disease, respectively [68,112]. In addition, it has become apparent that more than the two Th cell lines are implicated. Th17 and Tregs are also important players in the immunopathogenesis of IBD [5,56,182]. One observation is that the Th classifications of IBD are merging together. Strober & Fuss state that although both forms of IBD have overlapping genetic profiles, with nearly 30 genes conferring susceptibility to both CD and UC, the diseases are still characterized by different T cell responses [182].

Supported by several reports, the leading hypothesis is that CD is the result of a mixed Th1/Th17 response [149,156]. In contrast to CD the immunopathogenesis of UC has been more difficult to ascertain. Several studies have implicated Th17 cells in the pathogenesis of UC [66,97], whereas others have reported increased production of Th1 associated cytokine IFNG [12]. In addition, NKT cells producing IL13 have also been suggested as key players of UC immunopathogenesis [68]. The Th1/Th2 paradigm may therefore seem less well suited as a concept for understanding of IBD pathogenesis.

In **paper I** we investigated the cytokine gene expression profiles in the untreated mucosa of CD and UC patients, aiming to characterize potential differences between the two diseases and relate cytokine profiles to disease activity. This study showed that gene expression levels of *IL17A* and *IL23* were increased in both diseases, and also highly correlated to the disease activity. The expression of Th17 related markers were, in fact, higher in UC mucosa than CD mucosa, which is in line with the results of other studies [23,97]. We also found equal amounts of *IFNG* mRNA in CD and UC, which was in concordance with the results from the study by Olsen et al [137] who found no major differences in cytokine mRNA expression profile between untreated CD and UC patients. In our study (**paper I**) there was also positive correlation between cytokine expression level and disease activity for both UC and CD, with the exception of *IFNG* which surprisingly did not correlate significantly with CDAI score. According to our results the immune response for both UC and CD is characterized by a mixed Th1/Th17 response. The results presented in **paper II** support this as it was again demonstrated an increased mRNA expression level of *IFNG* and *IL17A* before initiation of anti-TNF therapy, and a positive correlation between these cytokines was observed (**paper II**). There were no significant correlations between mRNA levels of *IL17A/IFNG* and Th2 transcription factor *GATA3*, respectively, suggesting that there is a polarization between the Th1/Th17 and Th2 subsets. *GATA3* mRNA levels were significantly higher in UC patients compared with controls. It has been suggested that the immune response will differ depending on the phase of inflammation; a Th1 response characterizes the initial phase, a mixed Th1/Th17 response characterizes the advanced phase, and Th17 acts as an inflammatory moderator [182]. Sub-analyses on longstanding versus newly diagnosed IBD in **paper I** did not reveal any significant differences in cytokine expression profile, but it is not possible to identify initial and final phases of inflammation based on this material.

There is an ongoing discussion about the anti-inflammatory properties of IL17A. Results from the RCT on anti-IL17A monoclonal antibody for CD showed that inhibition of IL17A was ineffective [88]. The authors suggested that blocking of IL17A may interfere with

a protective function of IL17A in the intestine, and it was shown that rates of adverse events were higher than for the placebo group, especially for infections. In **paper II** we investigated whether the expression levels of cytokines from different lines of T cells could be utilized as markers of response to anti-TNF therapy. Surprisingly it was demonstrated that both elevated pre-treatment expression levels of *IL17A* and *IFNG* predicted remission after IFX therapy. There was also a significant positive correlation between them. A functional relationship between Th1 and Th17 cells has been suggested since the identification of Th1/Th17 clones that expresses both IFNG and IL17 with shared functional features [7]. It has also been demonstrated that Th17 deficient mice develop a more aggressive inflammatory disease, and that treatment with IL17A can modulate a Th1 polarization in vitro and down-regulate IFNG production [132]. In addition, a distinct population of Tregs that produce IL17 has been characterized [86].

It seems that the downstream inflammatory events are quite similar in UC and CD, although the initiating immune response might be different. In view of the increasing knowledge on T cell plasticity, i.e the ability of Th cells to convert from one subset to another [133], a complete understanding of the mucosal immune response in CD and UC might not be possible, and perhaps of limited clinical relevance. Clinical and immunological classifications have focused on anatomy and T cell lineages, but it has become apparent that there is a need to reclassify and subdivide IBD patients based on their natural disease course and pathophysiological mechanisms. A personal molecular profile at time of diagnosis based on serum, genetic profile and tissue markers is useful in a clinical setting and can facilitate tailored follow-up [200]. Identifying relevant markers and cytokine profiles that can be utilized for the purpose of IBD profiling is still important and there is a need for translational research on this field.

4.2.2 Utility of biomarkers

As mentioned in the introduction, biomarkers are characteristics that are objectively measured and can be utilized either for prognostic (clinical outcome) or predictive (therapeutic effect) purposes. There are very few reports on mucosal markers of clinical interest (for review see [61]). Mediators of inflammation in IBD, especially those correlating with the disease activity and targeted therapeutically with good results, are of great interest as possible predictors of therapeutic outcome.

Olsen and co-workers identified TNF as a marker of inflammation [137], and reported that elevated TNF was a predictive marker for non-response to IFX induction therapy [136].

Our study on untreated IBD and mucosal expression of Th1 and Th17 related cytokines (**paper I**) gave further characterization of the immune response in active UC and CD, and identified cytokines from the Th17 cell subset as markers of mucosal inflammation. In **paper II** we identified elevated expression levels of *IL17A* and *IFNG* as predictive markers of response to IFX therapy. Patients with high expression levels of *IL17A* and *IFNG* had increased odds of remission after induction therapy of IFX compared to low expression. Arijs and colleagues have performed gene array studies on UC [9] and CD [10] and identified gene panels that can predict response to IFX with high accuracy. In line with our results in **paper II**, the risk increasing IL23R genotype status has been identified as a predictor of early response to IFX [90], this genotype has been linked to Th17 cell function in another report [167]. In **paper III** the same hypotheses were tested in a cohort of CD patients treated with adalimumab; no predictive pre-treatment mucosal markers could be identified as far as predicting mucosal healing after adalimumab induction therapy. Arijs et al performed a gene profile study in CD. They found no predictors of non-response in ileal CD, whereas colonic CD behaved much as UC and a gene signature which predicted non-response 100% accurately was identified [10]. Gene expression profiling has become increasingly investigated for its utility as predictive biomarker. CD and UC are complex multi-genetic disorders. However, given the fact that there is a considerable amount of environmental and demographical contribution, it seems reasonable that a multivariate approach will increase the accuracy.

In **paper IV** we assessed the time to relapse after withdrawal of anti-TNF therapy in a cohort of CD patients with MH. We identified elevated mucosal expression levels of *TNF* and *IL17A* as predictive markers of early relapse after withdrawal of biologics. This is the first report on pro-inflammatory cytokine expression as biomarkers for relapse in CD patients. In clinical practice the utility of recognizable factors associated with low risk of relapse after drug withdrawal is obvious. MH is associated with longer relapse-free survival in CD patients during ongoing anti-TNF therapy [13], and after withdrawal of this treatment [108].

Biomarkers may have a role at nearly every point in the disease management, from diagnosis to predicting severe disease course, treatment failure and time to relapse. A biomarker is objectively measured and is of clinical value only if it is accurate and reproducibly obtained, it needs to be easy to interpret for clinicians and have a high sensitivity and specificity for the outcome it is expected to identify (for review, see [115]). Mucosal gene expression of inflammatory cytokines is one possible approach to assess the inflammatory activity. In **paper I** a characterization of mucosal immune response in untreated IBD was performed. The identified mediators of inflammation were further analyzed as possible

predictors of treatment outcome. *IL17A*, *IFNG* and *TNF* appeared to be promising candidate markers of inflammation, response to anti-TNF therapy and outcome after treatment discontinuation. ROC analyses were performed in **paper II** and **IV** in order to assess test characteristics for the identified predictors; none of the mucosal markers predicted outcome with impressive test performance. However, the results hold promise for the development of models that can identify patients in need of intensified or prolonged therapy (**paper II**) and patients that will relapse early after withdrawal of targeted therapy (**paper IV**). So far, no single marker has proven to possess all the qualities of an ideal marker. Identifying candidate biomarkers is only the first step towards implementation of biomarkers in clinical practice. Proper validation is the critical step [1]. We have identified some mucosal inflammatory mediators that are of great interest, but validation studies are needed. A combination of biomarkers might increase the strength of the analytical procedures. There is incomplete penetrance of the genetic phenotype, and the translational and functional aspects of the genetic abnormalities are far from resolved. Genetic, molecular, clinical and environmental markers and characteristics will have to be integrated, and could open for reclassification of IBD and individualized management.

4.2.3 Predicting and defining mucosal healing

Healed mucosa has been introduced as a therapeutic goal after the introduction of anti-TNF therapy, mainly because anti-TNF therapy made this achievable (for review see [198]). However, for CD the concept of MH is not fully defined and validations are lacking. The term has been defined differently in different studies [96,155]. This lack of a unified definition makes comparison of results from different studies on MH in CD somewhat problematic. For UC the term MH lacks a validated definition, but it has been generally accepted that an endoscopic sub-score of 0-1 defines healed mucosa. The limitations have been discussed previously.

In **paper III** we studied a population of patients with active CD treated with ADA to complete healing, defined as absence of any signs of mucosal inflammation. Complete endoscopic healing accumulated to 44% of patients after 52 weeks of therapy and was paralleled by a significant reduction of mucosal gene expression of several pro-inflammatory cytokines. The recently published EXTEND trial [155] demonstrated similar mucosal healing rates at week 12 as our results presented in **paper III**. The results are also in concordance with previous studies on IFX therapy in CD [34,151]. Compared to a group of control patients, however, mRNA expression levels did not decrease to normal levels in all patients in

our study. In fact, over 50% of patients still had elevated expression levels of *TNF* and *IL17A*. In **paper IV** the implication of this was further studied in CD patients with mucosal healing after anti-TNF therapy, and we found that there were significant associations between elevated mRNA levels of *TNF* and *IL17A* and early relapse after anti-TNF discontinuation. The association between these mucosal markers in healed mucosa and time to relapse has, to our knowledge, not been demonstrated before. Low pre-treatment *TNF* mRNA expression has been identified as a strong predictor of long-term remission in CD on continuous IFX therapy in a previous report [168].

The term deep remission in CD was recently introduced [192], defined as CDAI <150 and MH. As mentioned previously, however, there is no validated definition of MH, and the correlation between MH and transmural healing remains to be studied. Imaging techniques like MRI or CT can be used to assess disease activity in the different layers of the bowel wall, but validated definitions of transmural healing are still lacking. The essential question is whether complete treatment response not only should be assessed by MH, but also should include normalization of immunological parameters like gene expression of pro-inflammatory cytokines. In **paper IV** we introduced the term *immunological remission*, based on the finding of increased immunological activity in mucosa with healed appearance, and the increased risk of early relapse in these patients.

4.2.4 Therapeutic stop criteria

So far there is no general agreement on if and when anti-TNF therapy can be discontinued safely in IBD patients in remission. The results presented in **paper IV** show an overall relapse rate of over 70% in 1 year, in CD patients with documented MH who discontinue anti-TNF therapy. However, in patients with normalized gene expression levels of *IL17A* and/or *TNF*, over 60% were still in remission after 52 weeks. Risk of relapse was significantly higher in patients lacking normalization of pro-inflammatory cytokines.

In a recent review it was stated that around 30% of CD patients are IFX dependent, and will relapse shortly after drug cessation [55]. The recent STORI-trial [108] assessed the risk of relapse after IFX therapy was discontinued in CD patients who had been on IFX therapy for at least one year, and were on maintenance therapy with anti-metabolites. 50% of patients relapsed within a year; risk factors for relapse were male gender, absence of surgical resection, elevated leukocyte count/CRP/fecal calprotectin and lower level of haemoglobin. Patients with more than 2 of these factors had a 15% risk of relapse within 1 year. An

increased endoscopic activity score (CDEIS) was also identified as a risk factor for relapse [108]. Interestingly, several of these predictive factors are markers of inflammatory activity, thus indicating that true remission may not have been obtained. Waugh and colleagues [206] followed a cohort of CD patients after IFX discontinuation from remission (defined as steroid-free remission > 6 months) to relapse. They found a long-term remission rate of 35% after 7 years of follow-up, no predictors of relapse were identified [206]. A recent Danish study [180] on IFX discontinuation in both forms of IBD demonstrated maintained remission of 61% in CD and 75% in UC patients who were in steroid-free remission at time of discontinuation. No risk factors of relapse were identified. We found no other risk factors for relapse than the mucosal immune markers. This could in part be explained by the small sample size and lack of power, but data supporting the use of clinical markers as predictors are scarce [115]. In addition, most of the clinical factors mentioned in the STORI-trial were within the normal range in our material at time of discontinuation (**paper IV**).

How can we identify patients that will remain in long-term remission after discontinuation of anti-TNF therapy? In clinical practice the utility of recognizable factors associated with low risk of relapse after drug withdrawal is obvious. Documentation of MH has become a critical component of outcome measurement. We know from several studies that MH is associated with favourable prognosis [13,33,170]. Normalization of inflammatory mucosal markers that are known mediators of the immune cascade in IBD can be a valuable addition to a more complete definition of the term MH.

The London position statement [42] concerning the therapeutic approaches of IBD management, recommended the following after reviewing the current evidence on IBD treatment: patients who respond to induction treatment with anti-TNF will benefit from systematic re-treatment, and there are insufficient data to make recommendations on when to stop anti-TNF therapy. English guidelines (NICE) regarding treatment of CD recommends that withdrawal of anti-TNF therapy is considered for all patients who are in stable clinical remission [127]. Increasing expenses in IBD management makes it necessary to identify patients where anti-TNF therapy can be stopped.

Over the past few years several studies have investigated the effect and safety of reintroduction of anti-TNF therapy after a period of withdrawal. Lack of immunomodulators and/or pre-treatment with steroid was associated with acute infusion reaction when reintroducing anti-TNF after a period of withdrawal [51]. No significant differences in adverse events or secondary loss of response were demonstrated in the same study. The STORI trial found that re-treatment with IFX was effective and well tolerated in 88% of

patients who experienced a relapse after discontinuation of IFX [108]. Unpublished data from our ongoing IBD study are in concordance with these results.

5. Conclusions and implications

5.1 Conclusions

- Increased levels of *IL17A* and *IL23* mRNA were found in both UC and CD. Association to the grade of inflammation and clinical activity was also observed. *IL17A* and *IL23* mRNA were significantly higher in UC than in CD. Th1 and Th17 cytokines seem to act synergistically in inflammatory bowel disease (IBD) with no apparent polarization between UC and CD.
- High expression of Th1 and Th17 related cytokine genes in the mucosa of UC patients could potentially predict a favourable outcome of IFX induction therapy. Th2 and Treg related mediators did not appear useful as predictive markers in UC. No association between expression levels of mucosal markers and response to therapy could be demonstrated in CD patients.
- Normalization of mucosal gene expression of cytokines did not occur in all CD patients with endoscopy-verified healed mucosa.
- Mucosal healing and normalization of *TNF* and/or *IL17A* mRNA expression predicted long-term remission after withdrawal of targeted therapy in CD patients.
- To optimize treatment of IBD we should aim to develop new algorithms that implement the knowledge of basic IBD immunopathology

5.2 Implications

5.2.1 Clinical implications

CD and UC are severe, lifelong diseases that impact patients and society, quality of life and health care costs. The advent of targeted therapy has improved IBD management.

Nonetheless, not all patients require long-term IFX or ADA therapy. There is a need for identifying useful biomarkers that can be utilized for tailoring and optimizing IBD treatment.

We have identified several mucosal markers that appear promising as predictors of response to anti-TNF therapy, and more importantly as predictors of outcome after discontinuation of therapy. In a model with clinical and demographical factors that are known to be associated with certain disease phenotypes and clinical outcome these mucosal markers could be a valuable addition that could potentially increase the accuracy of prediction. Based on the data

discussed above we suggest immunological remission, defined as MH in addition to normalization of pro-inflammatory cytokine expression, as a feasible stop criterion when treating IBD patients with anti-TNF therapy.

5.2.2 Research implications

We have identified inflammatory mediators that appear to be common for both CD and UC, possibly as mediators downstream in the inflammatory cascade of IBD. These pro-inflammatory mediators seem to be promising candidate predictors for therapeutic and clinical outcome, and illustrate that basal research can be clinically useful in very direct ways. Further research is needed to validate and establish biochemical, immunological and epigenetic biomarkers of clinical value, such as predicting severe disease course and effect of high cost targeting therapy. Markers that can be used as criteria to stop treatment without increasing risk of early relapse should be identified. Moreover, we should identify surrogate markers for immunological remission, as the PCR method is cumbersome and expensive in a clinical setting, and not available in every institution. Proper and extended validation studies are essential in translational research in order to confirm the results mentioned above.

It is of essence to continue the effort to reveal the underlying pathogenesis and characterize the immunological and genetic basis of IBD, as this will help identify new targets for therapeutic intervention. One of the greatest challenges is the lack of knowledge on which and why primary events initiate the inflammatory process. Genetic abnormalities have been identified, but the functional consequences are yet to be determined. Future research must deal with the complexity in multimodal measurements; the so-called *systems biology* approach. This strategy integrates data from different “omics” technologies, such as transcriptomics, epigenomics, proteomics, and metabolomics, in order to identify mechanisms and pathways of pathogenesis. IBD research thus requires a multifaceted and integrated approach. Understanding the structure and function of each protein and the complexities of protein–protein interactions in addition to metabolic end products of cellular processes will be useful for developing effective diagnostic techniques and targeted therapy in the future. Functional genomics techniques could identify useful biomarkers for early diagnosis, disease classification, and a tailored therapeutic approach.

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Appendix

Paper I-IV

Paper I

Paper II

Paper III

Paper IV



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