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When is remission remission?

Elucidating the remission state in Ulcerative Colitis: a multimodal exploration

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Summary

Ulcerative Colitis (UC) is a chronic, inflammatory disease of the colon that has a relapsing-remitting characteristic. The disease management consists of prolonging periods of remission and reducing relapse frequency. There is currently no universally accepted definition of remission in UC. There are different methods of establishing if a patient is in remission, but the lack of definition and knowledge make it difficult to know which method to use. The majority of these methods are poorly described for remission patients representing a substantial knowledge gap.

This thesis explored the remission term by investigating the mucosal transcriptional profile in UC remission patients and the utility of histology and transcripts as evaluation modalities. The project was a part of the Advanced Study of Inflammatory Bowel disease (ASIB) prospective study at the University Hospital of Northern Norway, Tromsø. All patients were recruited from August 2013 to April 2016 and they have given written consent to participate in the study.

We found that several of the gene transcripts investigated were differently expressed in UC remission patients in comparison to healthy controls. These genes were largely related to pro-inflammatory mechanisms and barrier dysfunction, indicating that despite an apparent normal mucosa in endoscopy, the mucosa differed on a transcriptional level. We then investigated if histology could detect inflammation and consistently classify a patient as in remission using the different scoring indices. The results showed that histology could detect inflammation invisible to the naked eye, but the histologic scores varied too much to be accurate in a categorical classification. The main source of variance was the histologic raters. Lastly, we investigated if any of the clinical, endoscopic, histologic, or transcriptional variables could predict impending relapse. The results showed that patients with a low ratio between two transcripts had 5.3 times higher risk of relapse. Histologic factors did not turn out to be predictive of relapse. Clinical and endoscopic factors were promising but ultimately not significant, possibly hindered by low pow er.

The conclusion was that several gene transcripts were differently regulated in UC remission patients compared to healthy controls. Some of these may be able to predict relapse and have potential use as biomarkers to improve the current treatment regimes. Histology might be used as an aid in tailored treatment but should not be used as a hard endpoint due to high variance. The transcript ratio must be further validated before implementation into clinical practice.

Sammendrag

Ulcerøs kolitt (UC) er en kronisk, inflammatorisk sykdom i tykktarmen som har uregelmessige perioder med høy og lav inflammatorisk aktivitet. Behandling forsøker å opprettholde lav-inflammatoriske perioder samt minimere antall tilbakefall til perioder med høy aktivitet. Periodene med lav aktivitet kalles remisjon og per nå finnes det ingen anerkjent definisjon av remisjon. Det finnes mange metoder å fastsette remisjon på, men mangel på definisjon og kunnskap gjør det vanskelig å vite hvilke som er nyttige. De fleste av disse metodene er dårlig beskrevet hos remisjons pasienter, noe som utgjør et kunnskapshull.

Denne avhandlingen utforsker vi remisjonsbegrepet ved å undersøke tarmslimhinnes genutrykk (transkript) profil hos UC remisjonspasienter og sammenligner den med kontroller. I tillegg evaluerer vi verdien av histologi og transkripter i vurderingen av remisjon. Denne studien er en del av Advanced Study of Inflammatory Bowel disease (ASIB) prosjektet som går ved Universitetssykehuset Nord-Norge i Tromsø. Alle pasienter ble rekrutter fra august 2013 til april 2016 og har gitt skriftlig informert samtykke til å donere biopsier fra tarmslimhinnen.

Vi fant at flere gentranskripter var uttrykt forskjellig hos pasienter i remisjon av UC sammenlignet med kontrollene. Disse genene var i hovedsak relatert til pro-inflammatoriske prosesser og dysfunksjon i tarmslimhinnens barriere. Dette viser at til tross for en tilsynelatende normal tarmslimhinne ved endoskopisk undersøkelse så er det forskjeller på et transkriptnivå. Videre undersøkte vi om histologi kunne detektere inflammasjon og deretter presist klassifisere pasientene i remisjon med de mest vanlige skåringsindeksene. Resultatene viste at histologi kan oppdage inflammasjon som ikke ses ved vanlig endoskopi, men at det er for stor variasjon i de histologiske skårene til at de kan benytte til å klassifisere pasienter. Hovedkilden til variansen var de som vurderte de histologiske snittene. Til sist vurderte vi om noen av de kliniske, endoskopiske, histologiske eller transkripsjonsvariablene kunne identifisere hvem som fikk tilbakefall. Vi fant at et lavt ratio mellom to transkripter gav 5.3 ganger økt risiko for tilbakefall. Ingen histologiske variabler økte risikoen for tilbakefall. Kliniske og endoskopiske variabler var på grensen til signifikant, men kan ha blitt begrenset av et for lite datasett.

Konklusjonen er at det er flere gentranskripter er uttrykt forskjellig mellom pasienter i remisjon av UC og kontroller. Noen av disse transkriptene er assosiert med økt risiko for tilbakefall, og kan derfor bidra til bedre behandling av UC pasienter. Histologi kan være til hjelp i å skreddersy behandling til pasientene, men bør ikke være et behandlings mål i seg selv. Transkriptene må valideres i et eksternt datasett før det kan implementeres i klinisk praksis.

Abbreviations

5-ASA - 5-Aminosalicylic Acid
ACTB - Actin-Beta
HPRT1 - Hypoxanthine-guanine phosphoribosyltransferase
ADAM17 - A Disintegrin and Metalloprotease domain 17
APC - Antigen Presenting cell
ASIB - Advanced Study of Inflammatory Bowel disease
ATP – Adenosine Triphosphate
AUC – Area Under Curve
CASP8 – Caspase 8
CD - Crohn's Disease
CHUK - Component Of Inhibitor Of Nuclear Factor Kappa B Kinase Complex
CI – Confidence Interval
CLDN2 – Claudin 2
CRC – Colon-Rectal Cancer
CRP – C-Reactive Protein
C_T – Cycle Threshold
DEFB1 - Defensin Beta 1
DNA - Deoxyribonucleic Acid
dsDNA – Doubled Stranded DNA
ELISA - Enzyme-linked Immunosorbent Assays
ER - Endoplasmic Reticulum
FC – Faecal Calprotectin
FOXP3 - Forkhead Box P3
GATA3 - GATA Binding Protein 3
gDNA – Genomic DNA
GI – Gastrointestinal
GM-CSF - Granulocyte-Macrophage Colony-Stimulating Factor
GS – Geboes Score
GWAS - Genome-wide association studies
HLA - Human Leukocyte Antigen
IBD – Inflammatory Bowel Disease
IC – Interplate Calibrators
ICC - Intraclass Correlation Coefficient
IFNG – Interferon Gamma
IL – Interleukin
ILC – Innate Lymphoid Cells
IQR – Inter-Quartile Range
LASSO - Least Absolute Shrinkage and Selection Operator
LOA – Limits of Agreement
MES – Mayo Endoscopic Score
MHC - Major Histocompatibility Complexes
MIQE - Minimum Information for Publication of Quantitative Real-Time PCR Experiments
mRNA – Messenger RNA
NGS – Next Generation Sequencing
NI – Nancy Index
NPV – Negative Predictive Value
OS – Oxidative Stress
PPV – Positive Predictive Value
PRO - Patient Reported Outcome
PROM - Patient Reported Outcome Measures
qPCR - Quantitative Polymerase Chain Reaction
RG – Reference Gene
RHI – Robarts Histopathological Index
RIN - RNA Integrity Number
RNA - Ribonucleic Acid
ROC - Receiver operating characteristic
RORGT - RAR-related Orphan Receptor Gamma
RPLP0 - Ribosomal Protein Lateral Stalk Subunit P0
SCCAI - Simple Clinical Colitis Activity Index
SDI - Sociodemographic Index
SPI1 - Spi-1 Proto-Oncogene
STAT - Signal Transducer and Activator of Transcription
TBX21 - T-Box Transcription Factor 21
TFF3 - Trefoil Factor 3
TGFB1 – Transforming Growth Factor Beta 1
TH – T-helper
TNF – Tumour Necrosis Factor
TRAF1 - TNF Receptor Associated Factor 1
Treg – T-regulatory
UC – Ulcerative Colitis
UCCIS - Ulcerative Colitis Colonoscopic Index of Severity
UCCS – Ulcerative Colitis Clinical Score

ACTB = Gene
ACTB = Protein

List of papers

1. Arkteg CB, Goll R, Gundersen MD, Anderssen E, Fenton C, Florholmen J. Mucosal gene transcription of ulcerative colitis in endoscopic remission. Scandinavian Journal of Gastroenterology. 2020:1-9
2. Arkteg CB, Wergeland Sørbye S, Buhl Riis L, Dalen SM, Florholmen J, Goll R. Real-life evaluation of histologic scores for Ulcerative Colitis in remission. PloS one. 2021;16(3):e0248224
3. Arkteg CB, Gundersen Dixon M, Anderssen E, Florholmen J, Goll R, IFNG:IL33 ratio predicts relapse in UC remission patients [Manuscript]

Introduction

1.1 Ulcerative Colitis

Ulcerative Colitis (UC) is a chronic inflammatory disease and is one of two diseases included under the umbrella term Inflammatory Bowel Disease (IBD). The other is Crohn's Disease (CD) which can affect the entire alimentary tract. In contrast, UC affects the colon only.

1.1.1 Epidemiology

UC has an increasing incidence world-wide ranging 1-24 cases per 100 000 and an aged-standardized prevalence of 7-422 per 100 000, depending on location (1, 2). The prevalence in some places in Europe surpass 0.3%. The disease has a peculiar distribution with higher numbers in the northern hemisphere compared to the south. After a decade of increasing incidence in the developed countries the curve now flattens. Aamodt et.al demonstrated that there is a higher prevalence in urban areas than in rural and that the disease is more frequent among higher educated people compared to those with the lowest level of education (3). A recent paper found a clear trend that countries with high Sociodemographic Index (SDI) have higher prevalence than countries with low SDI (2). An estimate from Burisch et al. suggests that IBD has a direct healthcare cost of 4.6–5.6 billion Euros/year (4), and this cost is likely to increase as more people are affected and new expensive treatments are introduced. There does not seem to be any sex predominance in UC (5).

1.1.2 Pathogenesis of UC

The pathogenesis of UC is described as multifactorial where environmental, microbial, genomic and immunological factors are of main interest (6, 7). In other words, UC is a disease where a dysfunctional immune system responds to a dysbiotic microbiome in a genetical susceptible colon. All these factors interact and affect each other resulting in a very complex pathogenesis.

1.1.2.1 Environmental factors

The increase of the disease and its epidemiological characteristics suggest a strong environmental relationship. The increase in the incidence of UC seen in industrial countries with rising life expectancy and improved sanitary conditions gave rise to the "hygiene hypothesis" (8). This hypothesis suggests that the decrease in enteric infections and general microbes that previously affected the intestines at a young age are necessary to prime the immune system to distinguish between commensal and pathological antigens later in life. The results from a case-control study from New Zealand support this hypothesis. The researchers found that having a vegetable garden in childhood is protective against developing IBD, as the garden functions as a surrogate marker of exposure to dirt and dirt bacteria (9). A way of investigating the hygiene hypothesis is to study migration from developing countries to industrialized countries where immigrants come from low affluent and low health care coverage to higher affluence and general health care coverage. A study from Sweden shows that first generation immigrants have decreased risk of developing IBD whereas in some groups the second generation has higher risk, suggesting that environmental factors play an important role early in life (10).

An interesting notion is the importance of early bacterial colonization of colon from vaginal delivery in relation to the risk of developing IBD. Studies have shown that there is a difference in the microbiome (composition of bacteria in an environment) in the gut between babies born vaginally and

babies delivered by Caesarion section up to 14 months after delivery (11). Especially the phyla Bacteriodetes has a delayed colonization and it plays an important role in priming the immune system(12). However, no association is found in a meta-analysis investigating the association between mode of delivery and risk of IBD (13).

Interestingly, the same factors may exert different effects in different geographical groups. A case-control study from Australia investigated the same risk factors in resident Australians, Middle Eastern migrants (MEM) in Australia and a Lebanese in Lebanon control group (14). The results showed that the same factors have opposite effects in the different populations. Antibiotics, for example has a protective function in the MEM group, whereas in the resident Australian group it is a risk factor. The authors attribute this to the difference in prescription pattern. In the Australian group, antibiotics could be a surrogate for decreased microbial diversity, whereas in the MEM group, antibiotics could be a surrogate for GI-infection and ultimately increased diversity. Microbial diversity is discussed more in-dept in the following chapter.

Other environmental factors that have an impact on UC are oral contraceptives, urban living, soft drinks and vitamin-D deficiency, all of them increasing the risk (15, 16), whereas breastfeeding, pets, and smoking are some of the factors reducing the risk of developing UC (16-19). There is evidence for a strong association between the environment and the development of the disease, and that some of these associations may involve the microbiome as a causative agent.

1.1.2.2 Microbial factors

Bacteria is a natural habitant of the gut and it can reach concentrations up to 10^{11} to 10^{12} cells/g of stool (20). The microbiome has a variety of functions such as educating the immune system, secreting digestive enzymes and repressing pathogenic microorganisms (21). It is estimated that the gut contains over 1000 bacterial species with dominance in the phylae Firmicutes, Bacteriodetes, Actinobacteria and Verrucomicrobia (21-23). The microbiome of IBD patients is often termed “dysbiotic” (21, 24, 25). It is an ambiguous term with no clear definitions but can be understood as microbial imbalance or maladaptation in the colon, which may be part of the pathogenesis. There is evidence for a dysbiosis in UC, but it is less clear whether this is causative or a consequence of the inflammation (26). Antibiotics are mentioned as a risk factor for IBD in the westernized population. One reason being the effect on the intestinal microbiota and a potential cause for dysbiosis (27-29).

A well-crafted study with a “multi-omic” approach (metagenomic, metatranscriptomic, proteomic, metabolomic and viromic) investigated stool samples collected every two weeks from 132 participants throughout one year, and reports small differences between UC patients and normal controls (30). This is consistent with previous research which reports that the majority of UC patients have a “normal” microbiome, and only a subpopulation of IBD patients differs from their controls (31, 32). In the sub-population a lower abundance and/or diversity in the phylae Firmicutes, and Bacteriodetes are observed, while there is an increase in the Gammaproteobacteria, Proteobacteria and Enterobacteriacea (21, 33-35). In addition, a shift from obligate anaerobes towards more facultative microorganisms in the IBD microbiome, has been reported (35).

The microbiological difference in subpopulations may be a result of the different states in UC, where patients are either in quiescent or active states of disease. A study investigating the dynamics of the microbiome found higher volatility in the microbiome composition in UC- stool-samples than in

samples from healthy individuals. It did not, however, correlate with f-calprotectin which was used as a surrogate for disease activity (32).

A confounding factor when evaluating the difference between states on a taxonomic level is the heterogenous functions of bacteria. Because of bacteria's ability to horizontally transfer genes between species, a species function can differ from person to person based on the composition of species in its environment. Therefore, a different approach to understand the role of the microbiome is to look at metabolic pathways rather than types of bacteria.

The butyrate metabolism in bacteria promotes T-regulatory cells (Treg) development in the gut and enhances the mucus production from goblet cells and strengthens the barrier functions (36, 37). UC displays reduced butyrate metabolism (38). A bacterial indol metabolite named indoleacrylic acid is a product of the tryptophan metabolism. It promotes intestinal epithelial barrier function and mitigates inflammatory responses. Microbes in IBD patients have reduced ability to metabolize tryptophan (39). Other community-level shifts in the metabolism are magnesium metabolism and oxidative stress resistance. A magnesium importing ATP-ase is enriched in IBD subjects relative to controls, which may explain the increased risk of magnesium deficiency in IBD patients (40, 41). Oxidative Stress (OS) has a vital role in the immune defence, and it is implicated in UC pathophysiology (42). Interestingly, the IBD-associated microbiome has enriched reductase enzymes on a community-level, giving it a selective advantage in an OS rich milieu (41). Functional analysis have found a greater abundance in the chemical carcinogenesis pathway which suggest that the microbiota may contribute to the increased risk of CRC seen in UC patients (43).

Fungi and viruses are also implicated in IBD, but their significance and contribution are still uncertain because of few studies and technical challenges (44). Nevertheless, it is an area that holds much potential and will certainly be explored in the future.

Bacteria in the human colon play a crucial role in both sickness and health. To elucidate their role in UC the scientific community must first agree on the best investigation approach to use. New technology has increased the number of methods to assess composition, abilities, and functions of the microbiome. This has made the results difficult to compare and assemble.

1.1.2.3 Genetic factors

UC is not a directly hereditable disease, but there is a genetic component to the disease. This is evident by the accumulation of the disease within families. Twin studies from Sweden and Denmark found a concordance of about 20% between monozygotic twins compared to 0-4.5% in dizygotic twins indicating a genetic factor for the disease (45, 46). A meta-analysis supports this view as it reports that 12% of UC patients has a family history of IBD (47). Interestingly, the incidence of familial IBD is higher with earlier debuts of the disease, suggesting there is a stronger genetic component in paediatric UC.

New technologies such as Genome-Wide Association Studies (GWAS) and Next-Generation Sequencing (NGS) have made it possible to prod deeper into the genetical background of UC. About 200 susceptibility loci for IBD are identified, where at least 23 loci are unique to UC (48, 49). Nevertheless, these susceptibility loci and genetic risk factors only account for 20-25% of the heritability (49).

Genes within several different cellular responses and IBD-related processes are identified. *IL23R, IL12B* and *IL10* are some of the genes found to be different in UC patients compared to controls, and they relate to adaptive immunity and its regulation (50, 51). *CDH1*, *HNF4A* and *GNA1* are genes found to regulate epithelial functions and have been associated with the disease (52, 53). Different human leukocyte antigen (HLA) complexes have also been associated with UC. Especially HLA-DR2 is thought to be significant for the Japanese population (54, 55). As HLA-complex encodes the Major Histocompatibility Complexes (MHC), it is crucial for regulation of the immune system. In broad term genes related to ER-stress response, epithelial restoration and cell migration are just a few of the processes that have risk loci (56).

Other facets of the genetics of UC are epigenetics. Epigenetic is the action of regulating gene expression by modification of its structure, rather than an alteration of the sequence. This regulation can be done through DNA methylations. A study has found that hyper-methylation of genes is implicated in homeostasis and microbe defence, and that hypo-methylation of genes is related to immune response in UC treatment-naïve patients (57). Methylation of specific genes has also been suggested as biomarker for cancer surveillance in IBD patients (58)

A challenge with genetic variants is that they do not necessarily result in a dysfunctional protein. Therefore, all genetic variants must be functionally tested in order to comprehend their significance. This is time consuming and sometimes not possible as there is no validated benchmark test that predicts functional outcome of gene variants. Overall, allele variants, loci and methylation-status are all of importance for the understanding of the pathogenesis of UC.

1.1.2.4 Immunological factors

When evaluating the role of immune mediators in UC it is difficult to separate cause and effect. Many of the agents presented here are important for the disease but may be a consequence of inflammation rather than a cause of the disease.

The primary defence of the colon is its mucosal layer and the epithelial barrier. Its role is preventing microbes from entering the mucosa while allowing immune cells to sample antigens in the colonic lumen for immune priming. The priming is necessary to ensure a targeted and swift action if an intrusion does happen. The epithelial barrier in UC is thought to be defect, thus giving rise to the "leaky gut" hypothesis. The barrier consists of several cell types such as enterocytes, Paneth cells and goblet cells. The cells are bound together with proteins allowing regulation of the permeability through the barrier. These junctional complexes are named tight-junctions, adherence junctions and desmosomes (59). Alteration in the composition of these complexes has been found in IBD. Especially Claudin 2 is thought to be a central factor in the dysfunction of tight-junctions in UC (60-62). It is also documented an altered composition in the monolayer as mucin producing goblet cells are depleted, and there is a reduced amount of mucin even in the un-inflamed mucosa (63).

The reduced barrier function will result in bacterial peptides entering the mucosa triggering an immune response. This response is thought to be an overreaction to commensal antigens. The inappropriate response is mediated by immune cells such as neutrophils, dendritic cells, T-cells, B-cells, and innate lymphoid cells. A difference in response has been suggested to be crucial between CD and UC, where CD is T-helper cell 1 (Th1) mediated while UC is T-helper cell 2 (Th2) mediated

(64-66). This partition is debated as other studies have not found any difference in the type of T-cell expression between the diseases (67, 68).

Innate lymphoid cells (ILC) are thought to be an early instigator of disease. They are in function and phenotype similar to T-helper cells but lack rearranged specific antigen receptors. ILCs bridge the innate and adaptive immune system, sensing environmental changes and responding by secreting cytokines that activate the adaptive immune system. They can also present antigens on MHCII-class proteins, but with lower efficiency than professional antigen presenting cells (69). Three main subsets of ILC reflecting the function for Th-subsets are identified (table 1) (70). Note that there is plasticity between the subset based on the cytokine environment.

ILC3 has been frequently investigated in animals and is thought to be central to UC pathology. It possesses both the ability to aggregate and to ameliorate inflammations. The pro-inflammatory mechanisms occur through production of the pro-inflammatory cytokines IL17, IFNG and GM-CSF in response to IL23 and IL1B (71, 72). There is also evidence for ILC3 to produce IL22 in response to IL18 and IL21, and thereby inducing production of mucin and pro-inflammatory molecules in epithelial cells (73-75). This response is protective in certain situations of colitis, but in situations of on-going inflammation it exacerbates due to increasing chemo-attractants and stress response in the epithelial cells (76).

Table 1 ILC overview

	Transcription factor	**Cytokine productions**
ILC1	TBX21	IFNG and TNF
ILC2	GATA3	IL4,IL5, IL9, and IL13
ILC3	RORC	IL17, IL22, GM-CSF, and IFNG

T-cells are mentioned as important players in the disease. Both CD4+ and CD8+ T-cells are found to be increased in IBD intestines (77). While CD8+ T-cells are the effector cells, CD4+ cells are most frequently investigated in IBD. Five subsets of T-helper cells are now identified and thought to be relevant in IBD (TH1, TH2, TH9, TH17 and Treg).

TH1 is important in the protection against infectious pathogens, and they produce mainly IFNG and TNF activating macrophages and cytotoxic CD8+ cells in an IL12 dominant environment (78). TH1 cells are considered to play a more important role in CD pathology than in UC pathology. This is evident as there are small amounts of IFNG and its transcriptions factor (TBX21) in UC. In addition, treatment with anti-IFNG has not been successful in UC (79-81).

TH2 cells are in charge of defending the host against helminth infections (82). UC is described to be atypical-TH2 dominated. Atypical, because of the lack of IL4 presence, which is considered a typical TH2 cytokine. Nevertheless, the presence of IL5, IL13 and its transcription factor GATA3, suggest that TH2-like cells are major players in UC (61, 65, 83). TH2 immunity also plays a role in other diseases such as asthma and atopic dermatitis.

TH9 is a novel contributor to UC pathology. It is recognized by its transcription factor SPI1 (PU.1) and expression of IL9 cytokine (84). IL9 is found to affect the epithelial barrier negatively by disturbing tight junction proteins and preventing epithelial regeneration (85-87). It is believed that IL9 plays a role in the immunity against helminth and tumours (88).

TH17s' role in IBD is difficult to ascertain due to its plasticity and its dichotomous role in IBD. It is found to be increased in UC patients in comparison to healthy controls (89). It is identified by its main transcription factor RORC and its IL17 secretions. It is induced by TGFB1, IL1B and IL6, and it requires IL23 to maintain its differentiation (90). GWAS studies have found genes related to TH17 to be enriched in IBD (48). It exhibits both tolerogenic and pro-inflammatory properties. TH17 can in presence of certain infections transform into TH1 and produce IFNG. Conversely, during resolutions of inflammations it can transdifferentiate into IL10 producing Tregs (91). These multiple abilities make it difficult to investigate TH17, but all evidence point to it being a substantial player in the UC pathology.

T regulatory cells (Treg) are anti-inflammatory players in UC. It is a CD4+ immune cell identified by transcription factor FOXP3. Its role is thought to dampen inflammation through production of the anti-inflammatory IL10, which supresses the development of pathogenic macrophages and TH1 cells (92, 93). Another cytokine central to Tregs is the anti-inflammatory cytokine Transforming Growth Factor Beta 1 (TGFB1). It is produced by mature Tregs and induce Treg differentiation, as well as inhibit differentiation of T- and B-cells (94, 95). In addition to the anti-inflammatory effect, it is found to contribute to fibrosis in UC (96, 97). Consequently, it is proven to be upregulated in UC patients compared to controls (98).

Central Cytokines

The immune cells exert some of their effect through signalling molecules called cytokines. Therefore, some of the central cytokines for UC will be reviewed.

Tumour necrosis factor (TNF) is a central agent in immune defence and inflammation. Its role is primarily pro-inflammatory as it is secreted by innate defence cells such as dendritic cells and macrophages. TNF activates adaptive defence cell as well as promoting other pro-inflammatory effects such as impaired barrier function, angiogenesis and hypervascularization (99). It also exerts its effect on effector T-cells where it prevents apoptotic signals and thereby prolonging their effective periods (99). A testament to the importance of TNF is the effect of anti-TNF treatment on UC patients, where it is found to lower colectomy rates and steroid free remissions (100, 101).

IL1B is a part of the IL1 family of cytokines (other members are IL1A IL18, IL33 and IL36) and it is primarily thought to be proinflammatory in IBD. In UC, it is found a lower ratio of the IL1-R antagonists to IL1, which indicates increased signalling and effect of IL1 (102). In an animal model with *H. hepaticus*-triggered intestinal inflammation IL1B augments recruitment of granulocytes and activated ILCs. IL1B is also thought to be induced by Pathogen Associated Molecular Patterns (PAMP) signalling through a cytosolic molecular complex called NLRP3 inflammasome (103).

IL6 is another important proinflammatory cytokine. It is expressed in both myeloid cells and acquired immune cells such as Th2 and Th17. As TNF, it shares the effect of inducing other pro-inflammatory cytokines and preventing T-cell apoptosis, but unlike TNF, trials with anti-IL6 agents have not proven efficient as IBD treatments. The anti-IL6 drug Tocilizumab has showed efficacy in TNF refractory moderate to severe CD patients but struggled with high rate of adverse effects such as abscess formation and perforation (104, 105).

IL10 is one of the central anti-inflammatory cytokines. It exhibits its effect by suppressing pro-inflammatory production by Antigen Presenting Cells (APC) and inducing anti-inflammatory macrophages (106, 107). Increased levels of serum IL10 are identified both during active and inactive UC, suggesting that it is not able to contain the inflammation in the active phase, but is necessary for resolution of the disease (98, 108). Association between an IL10 single nucleotide polymorphism and IBD has been identified in a GWAS study, underlining the importance of IL10 for disease resolution (48).

IL23 belong in the IL12 family and shares a common subunit (p-40) with IL12. It is considered proinflammatory and IL23 is necessary to stabilize and maintain Th17-cells. IL23 is produced by sentinel tissue such as dendritic cells and macrophages in contact with antigens from the microenvironment. It augments TNF production and induces ILC3 to produce IL17 family cytokines (IL17A/F and IL22). Ustekinumab is a drug for induction and maintenance treatment and it targets the p-40 subunit and thereby blocking both IL23 and IL12 (109). There is currently no pure anti-IL23 agent approved for UC, but several trials are ongoing (110).

IL33 is a part of the IL1 family and it is a new and interesting cytokine in UC. IL33 has been proposed to have dualistic properties. It is found to be upregulated in inflamed mucosa especially in epithelial cells (111-113) and believed to function as an “alarmin” that rallies the colonic immune-defence (114). In contrast, it is also believed to ameliorate the inflammation through macrophage modulation (115) and is found to be up regulated in remission compared to healthy individuals (81).

TGFB1 is an anti-inflammatory and pro-fibrotic cytokine produced by mature Tregs. Its overall function is to suppress immune response to the luminal microenvironment and promote immune tolerance (94, 116). TGFB1 is also found to promote collagen production by myofibroblasts (117) There is an increased expression of TGFB1 in active UC (118, 119). Interestingly, TGFB1 is found to enhance barrier function through regulation of epithelial tight-junctions via maintenance of Claudin-2 and Occludin levels (120). Several animal knockout models have proved the negative consequences of not having TGFB1 (121)

It is worth remarking that although there is a strong focus on a single cytokine or cell, the UC pathogenesis is far too complex to be explained by one immunological factor only. Historically, the technology has limited the research to focus on one subject at the time. With the introduction of high throughput sequencing and mass spectrometry, it is now possible to investigate a more complex set of factors. This will be of great aid in the further unravelling of the UC pathogenesis.

1.1.3 Symptoms and signs of UC

UC affects primarily the colon and the main symptoms are bloody stools, abdominal pain and diarrhoea. About 6-30% of UC patients may also have extra intestinal manifestation such as primary sclerosing cholangitis, ankylosing spondylitis, and iritis/uveitis (122, 123). After onset of symptoms, UC usually progresses over several weeks and can lead to fatigue, weight loss and anaemia. There are several clinical scoring methods to help evaluating the severity of the disease. The Mayo clinical score (Table 2) was developed during a drug trial, and a high score when diagnosed is a negative prognostic factor (124, 125). The natural course of the disease is relapsing-remitting which means that there are periods of few symptoms alternating with periods with more severe symptoms. The Ibsen study identified different clinical courses for UC, where the majority experiences severe symptoms early in

the disease and milder symptoms as time progressed (126). Unfortunately, 37% of UC patients in the study experiences a relapsing-remitting course continually throughout the follow-up time of 10 years.

1.1.4 Treatment and clinical outcome

Clinical courses of UC tend to get less severe over time, but there are two severe outcomes, colectomy and Colon-Rectal Cancer (CRC). About 15% of all patients with UC ends up with a colectomy, and the major reasons are chronicity and uncontrollable inflammation (127). The risk of CRC has with modern treatment and follow-up programs been substantially reduced and is approaching the general background risk for CRC (128, 129). However, in certain subgroups like those with long duration, primary sclerosing cholangitis, and uncontrolled inflammation the risk is still elevated (130). The treatment for UC is a step-wise procedure where the clinical response determines if escalation to more potent drugs is necessary. The extent and severity of the disease will guide the physician in choice of medication to start with. The first step for induction of remission in mild to moderate UC is 5-ASA. Both oral and topical formulations are available, but about one third of the patients does not respond to 5-ASA alone (131). With lack of improvement, a step up to oral steroids such as Budesonide is recommended (132-134). In patients with moderate to severe UC, oral systemic steroids such as Prednisolone is appropriate (132, 133). About 16-33% does not respond to steroids and some become steroid dependent (135, 136). In patients who are refectory to steroids or have become steroid dependent, addition of thiopurines or, alternatively, a switch to anti-TNF treatment in combination with thiopurines or methotrexate is recommended (132, 133).

Although anti-TNF treatment has greatly improved the patient care, about 30-50% of UC patients does not respond to anti-TNF treatment and about 30% loose the effect after the first response (137-139). There are other biological drugs such as anti-A4B7-intergrins (Vedolizumab), anti-IL12/IL23 subunit (Ustekinumab) and antibodies targeting the JAK/STAT pathway (Tofacitinib) (109, 140, 141).

After induction of remission, maintenance treatment is needed to avoid relapse. 5-ASA is the first choice followed by thiopurines and/or anti-TNF treatment (132-134). There is currently no recommendation of treatment length for any of the medications used in maintenance treatment. One study has investigated the relapse rate for people in long term remission (> 1 year) on Mesalazine maintenance treatment. They found a need to maintain treatment up to 2 years after induction of remission, but after 2 years there is no difference in relapse rate between drug and placebo (142).

Treatment strategies are treat-to-target and the target is remission. What constitutes remission is heavily debated and is reviewed in separate chapters, but in general terms it is normalization of bowel movement, no rectal bleeding and near to normal endoscopic appearance of the mucosa (143, 144). Currently about 43% has a relapsing and remitting course during the first year after diagnosis and 16% has relapse after cessation of biological treatment (145-147). In a 10-year perspective about 67-83% relapses depending on the clinical situation (126, 148). This indicates a potential for improving disease management, and one way of improving is to have a clearer target, i.e. a better definition of remission.

Another important aspect of UC treatment is the medical non-responders. With increasing numbers of possible therapies there is a search for markers that can identify those who will benefit from a certain medication and those who are at risk of severe outcome and complications. This approach is termed personalized medicine and aims to tailor treatment to the individual patient based on multidisciplinary (multi-omics) data (149, 150).

1.2 Remission

1.2.1 Remission definitions

UC is a disease with periods of active disease with heavy symptoms, followed by periods of fewer symptoms. The later periods are labelled remission, a term without a clear definition. As knowledge of the disease and technology have progressed, the use of the term remission has changed. Initially, the term for symptomatic improvement or relief, as it was the best one could achieve with the drugs at that time. With new drugs and better endoscopic equipment, normalised mucosal appearance has been established as a positive predictor of outcome and was included in the remission evaluation (151). This gave rise to a new term called "mucosal healing", meaning an endoscopic near to normal mucosal appearance (152). Lately, several new remission terms are introduced, such as "deep remission", "immunological remission" and "histologic remission" (153, 154). One reason for the new ways of defining remission is the discovery of new modalities for predicting a beneficial outcome of the disease. As endoscopy proved to benefit the UC management other modalities such as histology, serum markers or genetical markers could lead to further improvement. With each of these new modalities follow multiple new scoring indices to aid the evaluation. Therefore, it is no surprise that confusion arises and consequently heterogeneity in the use of the remission terms. Boal et al. report this heterogeneity and attribute it partly to the large number of different scoring indices for UC (152). Ma et al. highlight in their systematic review of 83 randomized controlled trials (RCT), that the heterogeneity makes it difficult to compare trial results as 50 different definitions of remission or response were applied(155).

Another side of the remission debate is the issue of clinical-dependent remission criteria, meaning that different settings may have different markers for remission. A patient with ongoing biological treatment may have one marker for long term remission and thus a physician can de-escalate treatment, while another patient who is already in remission may have another marker for imminent relapse and should therefore prophylactically escalate treatment. Both these markers need to be considered when defining remission.

In the following sub-chapters important remission factors within each modality, including the most acknowledged scoring indices, will be reviewed. The remission term will exclusively be used for the clinical setting of de-escalation of treatment while others will be mentioned as relapse markers.

1.2.1.1 Symptomatic factors

The disease presents itself through symptoms, and for the patients the symptoms are their main concern and meter for disease activity. Therefore, any definition of remission must be founded on absence or minimal amounts of symptoms. For a disease with several symptoms, it may be difficult to ascertain which symptoms to include in the remission evaluation. If too many symptoms are included, the remission state will be unachievable and lead to over-medication. To include too few or wrong symptoms will lead to undertreatment. Currently, the main symptoms for UC activity evaluation are stool frequency and blood in stool. This is reflected by the fact that most guidelines recommend a normalization of these symptoms as a minimum remission criterion (132, 134, 156). Consequently, the three most applied symptomatic scores, Ulcerative Colitis Clinical Score (UCCS), Simple Clinical Colitis Activity Index (SCCAI) and

Mayo Clinical Score (Mayo score is a composite score for both endoscopic factors and symptoms, but its widespread use warrant inclusion), evaluate these symptoms (125, 157, 158). Scoring indices are applied to simplify the evaluation by making a patients' subjective symptoms more objective in order to establish a discerning cut-off value between active and remission state. Interestingly, in all three scores only two symptoms are assessed, stool frequency and rectum bleeding. Likely, to amend for limitation of evaluating a complex disease by only two symptoms, two of the three scores have a subjective "physicians' evaluation" grade. It is not until recently that Patient Reported Outcome (PRO) has become a focus and a source for evaluation of treatment response and endpoint in trials (159). Patient Reported Outcome Measures (PROM) is a tool developed to circumvent the physician subjective interpretation of the patients' symptoms. There are few PROMs developed for UC and even fewer are commonly applied, but Bodgers IBD-control is a PROM simple to use in clinical practice (160).

Table 2 Mayo clinical score

Stool frequency	
Patient reporting a normal number of daily stools	0
1-2 more stools than normal	1
3-4 more stools than normal	2
≥5 more stools than normal	3
Rectal bleeding	
None	0
Blood streaks seen with stool less than half of the time	1
Blood with most stools	2
Pure blood passed	3
Endoscopic findings (MES)	
Normal or inactive colitis	0
Mild friability, erythema, decreased vascularity	1
Friability, marked erythema, absent vascular pattern, erosions	2
Ulcerations and spontaneous bleeding	3
Physician global assessment	
Normal	0
Mild colitis	1
Moderate colitis	2
Severe colitis	3

1.2.1.2 Endoscopic factors

There are discrepancies between the symptomatic scores and endoscopic findings (161-163). Despite being almost asymptomatic, many patients display signs of inflammation in the colon. "Mucosal healing" is a term for mucosal appearance during endoscopic investigation. It is most often defined as Mayo Endoscopic Score of 0 or 1 (MES, Table 2).Shah et al. report in a meta-analysis that «mucosal healing» has improved outcome versus patients with endoscopic signs of inflammation (164). From the plethora of endoscopic indices the most applied for UC are the endoscopic component of the Mayo Score and Ulcerative Colitis Endoscopic Index of Severity (Table 3), but only the latter is validated (125, 165). There is one other validated score, the Ulcerative Colitis Colonoscopic Index of Severity (UCCIS), but it is not widespread in the clinical practice due to its complexity (166). There are differences between the scores, but all of them evaluate vascular pattern, ulceration/erosion and bleeding. In a remission setting most scores allow minor feature of inflammation such as a MES of 1 or a UCCIS of 2. There has been a recent change towards only allowing a remission patient to have a MES score of 0 as it has better outcome (167, 168).

Subjectivity in evaluation will challenge reliability and coherent rating in endoscopy. Features representing severe inflammation prove to be easier to evaluate than near to normal mucosa (169, 170). Therefore, the evolution of remission investigation needs to delve deeper into the mucosa in order to discover signs of inflammation invisible to the naked eye.

Table 3 The Ulcerative Colitis Endoscopic Index of Severity*.

Descriptors	Likert Scale anchor point	Definition
Vascular pattern	0: Normal 1: Patchy obliteration 2: Complete obliteration	0: Normal vascular pattern with arborisation of capillaries clearly defined
		1: Blurring or patchy loss of capillary margins
Bleeding	0: None 1: Mucosa 2: Luminal mild 3: Luminal moderate or severe	1: Some spots or streaks of coagulated blood on the surface of the mucosa.
		2: Some free liquid blood in the lumen
		3: Frank blood in the lumen ahead of endoscope or visible oozing from a haemorrhagic mucosa
Erosions and Ulcers	0: None 1:Erosions 2: Superficial ulcer 3: Deep ulcer	1: Tiny < 5 mm defects in the mucosa, of white or yellow colour with a flat edge.
		2: Larger > 5 mm defect in the mucosa, which are discrete fibrin-covered ulcers in comparison with erosions but remain superficial.
		3: Deeper excavated defects in the mucosa, with a slightly raised edge.

*maximum score = 8, scoring is based on the most severe area

1.2.1.3 Histological factors

Histology has a long history in the diagnosis of UC, and the earliest score was developed by Trulove and Richards in 1956 (171). Therefore, it is surprising that histology is not more often included in the daily clinical practice of UC. Many guidelines identify histology as valuable in disease evaluation, but with the current levels of evidence, it is not recommended as a treatment target or a factor for remission (132, 144). This is likely to change, as several histological features recently have been found to increase the risk of relapse. Both the American and European drug authorities require histology as an endpoint in clinical trials. The features that best predict relapse in patients with quiescent disease are increased neutrophils in lamina propria, basal plasmacytosis and mucin depletion (172-174).

As with the previous modalities there is no definition of histologic remission, nor is there a consensus of which scoring indices to use. Consequently, there are different remission definitions across the different scoring indices. The three most applied scoring indices are Geboes Score (GS), Robarts Histopathological Index (RHI) and Nancy Index (NI), and previous papers have reported remission cut-offs that span <2.1-3.1, <3-5 and 0-1, respectively (175-178). Despite having several different cut-offs histology has proved to be able to detect clinically relevant inflammation and predict relapse independently (179-182).

Drawbacks with histology are the slight increased risk of perforation under the procedure and the subjective nature of the image interpretations. RHI and NI are the only validated histological scoring indices and they have acceptable reliability and responsiveness (183). Interestingly, neither of them evaluates basal plasmacytosis or mucin depletions explicitly.

1.2.1.4 Serological factors

Both endoscopy and histology suffer from the same drawbacks, namely the cost and the risk of performing an endoscopy. Therefore, it is enticing to have a systemic marker, detectable in bloodwork for evaluation of remission. The only serological factor commonly used in disease evaluation is C-reactive protein (CRP), but it not recommended as a criterion for remission. This is because of the low sensitivity to low grade inflammation in the colon (184, 185). At diagnosis and in severe cases CRP has proved to be useful in predicting outcome and response to medication (186-188).

With the advance of more sophisticated tools for analysing blood, composite panels with many different markers have emerged. Planell et al applied microarrays to identify gene transcripts which can divide patients into a high- and low-risk group at diagnosis (189). Biasci et al. applied the same method to develop a panel which correlated well with endoscopy and was sensitive to changes, thus potentially being useful in the clinical evaluation of UC (190). Buorgonje et al. applied enzyme-linked immunosorbent assays (ELISA) to identify a combination panel consisting of serum amyloid A (SAA), IL-6, IL-8, and Eotaxin-1 which reliably can predict endoscopic disease activity in IBD (191). Kalla et al. developed an oligo-protein panel which identified patients with increased risk of treatment escalation (192).

1.2.1.5 Faecal factors

As with serum markers, faecal markers are more readily available than endoscopy and can be performed to a fraction of the cost. Unlike serum markers, faecal markers have the added benefit of representing the GI-tract rather than being systemic, which gives a more direct insight to the situation in the lumen of the GI-tract. Faecal calprotectin (FC) is the most applied faecal marker. It is a calcium binding protein found in neutrophils. Inflammation of the colon elevates calprotectin and this is a good marker for distinguishing between inflammatory conditions and functional diseases (193). Unfortunately, it does not have the same characteristics when discriminating between active and remission stages of UC (194). It has been proposed as a surrogate marker for mucosal healing with acceptable characteristics regarding sensitivity and specificity (195, 196). There are conflicting results regarding calprotectin's' ability to predict relapse. Theede et al. found that FC with a cut-off >321mg/kg is predictive of relapse, whereas Zhulina et al. report that a doubling of FC levels between two tests taken 3 months apart is predictive of relapse (197, 198). A meta-analysis performed by Li et al. found significant heterogeneity in the cut-off value for FC but suggest that consecutive tests could be beneficial in predicting relapse. The lack of a validated testing regime with cut-off values prevents FC from being a part of the UC remission definition. In addition, FC is not disease specific and the amount can vary greatly depending on the passage time of the stool (199).

Other suggested faecal tests are lactoferrin and Faecal immunochemical test (FIT), but they are not as good in predicting relapse as FC (195, 200).

1.2.1.6 Transcriptional factors

With the technology to analyse gene transcripts becoming readily available, one can delve even deeper into the core mechanics of inflammation. Since endoscopy is routinely performed on UC patients. it is possible to get samples from the core of the disease both in quiescent and active disease. Therefore, there is a potential for transcript biomarkers to be disease specific and to have excellent test qualities. *TNF* transcript has shown promising results as it correlates well with the grade of inflammation, and normalization predicts long term remission in UC patients (201, 202). IL33 is another cytokine showing promising results as a biomarker for remission (111).

Due to the complexity of cell signalling and biomechanics it is unlikely for one transcript to solely predict remission or relapse. Planell et al. performed a microarray analysis on non-IBD, remission UC and active UC, and discovered several upregulated genes in UC compared to non-IBD and where a significant number of genes were differently regulated in UC remission (203). These genes were at large related to epithelial cell proliferation, resistance to apoptosis, stress and wound healing. Fenton et al. found a different transcript profile by investigating mucosal biopsies with next-generation sequencing (NGS) (204). They found, as Planell et al., that restoration and improvement in the epithelial and mucus layers define the remission state. In addition, they found downregulation of Toll-like receptors transcripts which suggests a more inert immune response. They imply that this could be a result of medication. Another study found that transcripts related to expression of *IL17A/F* and *IL21* are predictive of relapse (205).

Transcripts have the benefit of being a clear window into the cells and mechanisms of UC pathology. Although, in a pathophysiological investigation the information gathered form transcripts are limited by the post-translational processes, which changes the resulting protein. Therefore, any transcriptional finding should be validated on a protein level, by histochemistry or proteomics.

1.3 Summary of introduction

UC is a chronic disease of the colon. The pathogenesis is currently not known, but the disease is believed to have a multifactorial cause. There is no cure with except of removing the colon, but recent advancement in treatment algorithms and medication have lowered the colectomy rates and reduced the risk for colon cancer. Despite these improvements a large part of the UC population is still troubled by relapses. The current management suffers from lack of coherence in terms, definitions and modalities of evaluation, making it difficult for the clinician to provide the best care for the patient. There are factors across several modalities that can predict beneficial outcome for UC patients. Symptoms, endoscopic evaluation and faecal calprotectin are already included in clinical practice to a certain extent. Factors within other modalities like histology and transcripts lack sufficient evidence to be included. To address this, the remission state must be thoroughly described with the modalities in question.

The purpose of this thesis was to lay a foundation for a more coherent practice by describing some of the different aspects of remission. These aspects were the transcriptional remission profile, the reliability of histologic evaluation of UC remission samples and, ultimately, to evaluate if any of these factors could predict relapse.

2 Aim of the thesis.

2.1 Hypothesis

To be able to provide precision medicine for UC patients, a clear definition of remission is needed. The defined remission state should be a state yielding a high likelihood of long-term remission. Remission could be described by different modalities, the most interesting being transcriptional, histology and endoscopy. The working hypothesis was that a thorough description of the UC remission state across several modalities might give the patients a better prognosis by increased precision of their treatment.

2.2 Aim

Aim 1: To characterize mucosal transcript of UC patients in clinical remission

Aim 2: Evaluate the reliability of the three most applied histologic indices in patient with endoscopic defined remission.

Aim 3: Investigate if any clinical and histological factors and/or gene transcripts can predict relapse/remission.

3 Material and methods

3.1 Population

All the three papers included in this thesis have been part of the Advanced Study of Inflammatory Bowel disease (ASIB) prospective study at the University Hospital of Northern Norway, Tromsø. Every study participant gave a written, informed consent with the possibility to withdraw participation after inclusion. The studies and storage of biological material were approved of by the Regional Committees for Medical and Health Research Ethics, division North (REK Nord ID:2012/1349) and the biobank was approved by the Norwegian Institute of Public Health (04/01690 HOD).

The selected participants presented in Table 4 were previously diagnosed with UC according to diagnostic guidelines (156). Sample collection was performed at routine endoscopy for patients in remission from August 2013 to April 2016. Inclusion criteria were patients aged between 18 and 80 with clinical and endoscopic remission defined as Mayo Clinical Score/Ulcerative Colitis Clinical Score (UCCS) of 0 or 1 and Mayo Endoscopic Score (MES) of 0 or 1. Patients with a total Mayo score above 1 or rectal bleeding were not included. IBD medication was neither an inclusion or nor an exclusion criterion. Baseline information was collected from a questionnaire answered at inclusion or from a review of the patient's journal.

Table 4 Overview of study design. The 41 UC remission participants in paper 2 and 3 were the same and they were drawn from the original 48 participants in paper 1.

	Included	**Study type**
Paper 1	48 UC 24 Normal controls	Case-Control observation
Paper 2	41 UC remission	Cross sectional observation
Paper 3	41 UC remission -27 Non-relapse -14 Relapse	Retrospective case-control

A control group of 24 non-IBD participants screened with colonoscopy for colorectal cancer or mild gastrointestinal symptoms were included. Criteria for non-IBD controls were no diarrhoea or other irritable bowel symptoms, as well as a completely normal endoscopy, with no polyps in sigmoid and no hyperplastic polyps in rectum lager than 5mm. Only non-IBD participants with Geboes score of 0 were included.

3.2 Sample analysis

All patients went through endoscopy with biopsy collection for quantitative polymerase chain reaction and histology. The biopsies were collected from multiple sites in the colon.

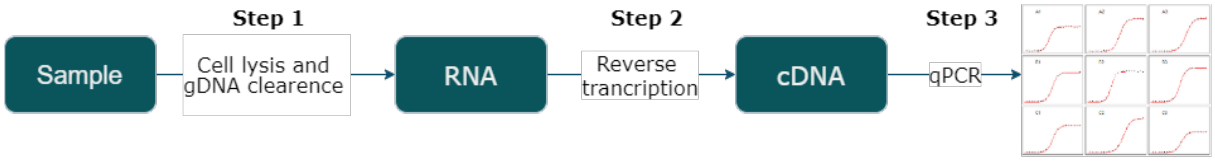
3.2.1 Quantitative Polymerase Chain Reaction

qPCR is a method that can detect, classify and monitor DNA and RNA targets. It is a relatively fast and cheap way to accurately quantify genes and gene expression, and it has a wide range of translational applications (for a review see (206)). When analysing gene expression, RNA is of interest and in cells it exits in different subtypes such as ribosomal RNA (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA). mRNA is the essential bridge between the genetical code (DNA) and the resulting protein and it is the target when preforming gene expression analysis. qPCR can detect mRNA transcripts in any biological sample if the transcript is known. However, there are both biological and technical limitations to the method which may challenge the accuracy of qPCR. The following sections presents the method in general and discuss its limitation.

Method

The analysis starts with extracting totalRNA (all types of RNA) form the biological sample and storing it. After the sample is extracted the RNA quality deteriorates rapidly, therefore the sample must be stored at -70℃ to maintain the integrity. Reverse transcription is the second step of the process and here the mRNA is copied in a proportionate amount to complementary DNA (cDNA). This product is the template used in the polymerase chain reaction (PCR) in step three. The PCR takes place in a thermal cycler that duplicate the template of interest and measure the amount. The thermal cycler works by heating and cooling the enzymatic reaction between DNA template and the polymerase in cycles. Before the cycle starts, there is an initial heating step where the solution is heated up to 95℃ for 2 minutes to separate secondary structures and activate Taq DNA polymerase. Thereafter, the cycles start by cooling the solution to 60℃ to allow for the primers and probe to anneal and elongate the primers with a strand complementary to the template. Next, the solution is heated to 95℃ in order to denature the newly synthetized double stranded DNA (dsDNA) into single stranded DNA (ssDNA). The solution then cools again, and the cycle is repeated 40 times and, if there are sufficient reagents, the amount of template is doubled after each cycle. The detection is done by registration of a fluorescent signal which is released in the presence of a specific gene sequence or dsDNA depending on method. If the gene of interest (GOI) is present it will for each cycle release a stronger signal which is proportional to the amount of template present (207). The cycle threshold (C_T) is the cycle for when the intensity of fluorescence signal reaches a set value. The more GOI in the sample the lower the C_T will be, since the threshold is reached with fewer cycles.

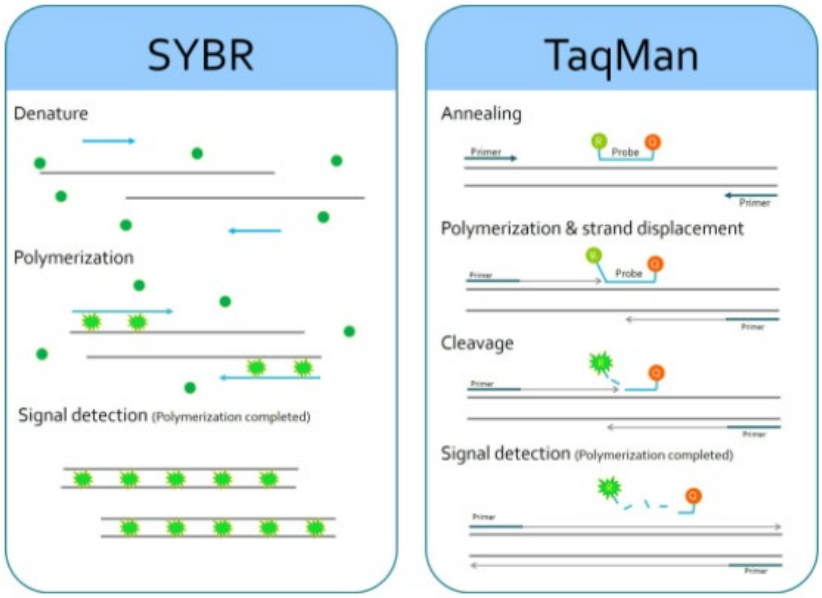
Figure 1 Procedural flow of a qPCR experiment.



There are different methods for detection of targets. Our laboratory applied both hydrolysis probe and SYBR-green. Hydrolysis probe is a technology where the probe sits between two primers and is conjugated with a reporter dye on the 5'end and quencher dye on the 3'end. The probe anneals with the targeted gene sequence on template DNA and the polymerase arrives to duplicate the target. The polymerase then dismantles the probe and thus releases the dye on the 5'end and distance it from the effect of the quencher. This removal allows the dye to release the fluorescence. The SYBR-green works by having a free-flowing dye which only binds to dsDNA and has a 1000-fold more efficient

fluoresces when bound. After each elongation in the PCR cycle SYBR-green attaches itself to the minor grove of the dsDNA and releases itself when the product denatured. The most important difference between the two methods is that SYBR green is cheaper and faster as it does not require a custom-made probe design. However, SYBR-green is less specific than the hydrolysis probe as it will bind to any dsDNA in the experiment, including misaligned primers and undesirable genomic DNA (gDNA). In addition, there is always background noise with SYBR-green analysis, therefore, the fluorescence signal will first be detectable when there are enough amplicons which releases the fluorescence. Despite these problems, studies have found that a well-designed and optimized SYBR-green assays can perform at the levels of the hydrolysis probe (208).

Figure 2. SYBR-green vs TaqMan. Illustrated difference between SYBR-green and hydrolysis probe (TaqMan). Printed with permission from SMOBIO Technology, Inc. Retrieved from https://www.smobio.com/faq-real-time-pcr "Real-time PCR Related Questions", 19.02.2021



Primers and probes

The internal validity is dependent on an optimal primer and probe design (assays). Assays can be designed in house through different software. It can also be bought from manufactures or be copied from previously published and validated assays. The hydrolysis probes were designed in-house with Beacon Designer v8 (PREMIER Biosoft International, Palo Alto, USA). To ensure specificity for mRNA, all probes spanned exon splicing sites. In addition, all primers and probes were run through Basic Local Alignment Search Tool (BLAST) to ensure specificity for the mRNA sequence in question (209). The efficiencies of all assays were measured by analysis of a dilution series standard curve made from cDNA from an actual biopsy. The SYBR green assays were ordered from Qiagen (Qiagen N.V, Venlo, Netherlands) and, consequently, the qualities of the SYBR Green assays were assured by Qiagen.

RNA quality

The quality of the RNA is paramount for accurate measurement of GOI. Sampling, storage, and extraction may affect the quality of the RNA. To preserve the quality as much as possible all biopsies

were immersed in RNAlater from Qiagen immediately after extraction. RNAlater is a solution that stabilize and protect cellular RNA in situ in unfrozen specimens. It postpones the need for freezing the samples which can be kept up to 1 week in room temperature (25ºC). RNA preservation was evaluated at our laboratory in 2006, and it was concluded that our handling of the samples exhibited minimal loss of RNA quality (210). RNA integrity number (RIN) is a scoring system that has been developed to report RNA quality. RIN over 5 indicates good quality and RIN over 8 is near to perfect total RNA (211). The average RIN value for a representable sample set from our population was 8.40 with a standard deviation of 1.67.

Reverse transcription

The conversion of mRNA to cDNA is a sensitive step in the qPCR process. It is important that the strategy and reaction conditions are the same in all experiments. To have an equal condition for all samples there must be an equal concentration of totalRNA in each reaction tube. Therefore, the totalRNA concentration and the calculated volume needs to be measured from each sample. Reverse transcriptions for the hydrolysis probe assays were performed with QuantiNova Reverse Transcription Kit, while, the SYBR-green assays utilized RT2 First strand Kit. Both kits were performed according to manufacturer's instructions.

Normalization

The qPCR method is limited by the uncertainty whether an observed difference is a consequence of true difference or error introduced by handling and preparation. When measuring genes, it is most common to measure it in relative amount to a known reference gene, so-called endogenous reference gene (RG) or housekeeping gene. This is necessary because it corrects for errors in the sample, introduced through handling, preparation and measuring. This correction is called normalization. The assumption is that a reference gene is stably expressed between samples and variations seen in this gene reflects technical imperfections only. The ideal reference gene is independent of any disease, condition, or external stimulus. Thus, candidate genes are often involved in basic and universal "housekeeping" cellular functions (212). However, there are studies suggesting that there are considerable variations even in these genes (213). Reference genes should therefore be validated for each experiment. In the present study *actin-beta* (*ACTB*) was applied for the hydrolysis probe experiments. *ACTB* had been previously used in our lab and had shown stable results with experiments with UC. For the SYBR-green experiments the geometric mean between *HPRT1* and *RPLP0* was applied (214). These later reference genes were found to have good stability value in an in-house validation study preformed with the normFinder method (215). The use of only *ACTB* in the hydrolysis probe experiment was a weakness and is not recommended by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. (216).

Reproducibility

As mentioned, all samples were run in duplicates, and if the difference was larger than 0.5 C_T values the sample was re-measured. Alle PCR runs had both positive and negative controls to check for contaminations. Interplate calibrator (IC) was the same as positive control and all plates were adjusted to the geometric mean of the ICs. Samples giving a measurable signal after 40 cycles were excluded as it implied a minute number of starting templates this implies, and a subsequent inherent risk of stochastic variability. This is in agreement with current guidelines (216).

Relative quantification and the comparative-method

C_T is the cycle number for when the fluorescence signal reaches an arbitrary threshold. The threshold is placed in the exponential phase of the amplification, and the same threshold must be used for all experiments. The numerical value of C_T is inversely related to the amount of amplicons in the reaction (217). There are primarily two ways to report qPCR data, by absolute quantification or by relative quantification. The absolute quantification allows for precise determination of copy per sample. It requires the construction of an absolute standard curve for each individual amplicon (218). In relative quantification the data is presented relative to another gene. The method is called comparative C_T or $2^{-\Delta\Delta CT}$-method. The method assumes that the efficiency of the PCR is close to one and that the efficiency of the GOI is similar to the reference gene (217, 219). The disadvantage of the absolute quantification method is the increased effort to make a standard curve. In most experiments it is sufficient to report a genes fold change rather than exact numbers. In papers 1 and 3 the comparative C_T-method was applied.

3.2.2 Histology

Histopathological samples were collected during the ASIB-study. In total 41 UC participants had samples of adequate quality to be included. The samples were evaluated by three pathologists, two general pathologists and one expert gastrointestinal (GI) pathologist. Two of the pathologists rated the samples on a white light microscopy, while the third evaluated them digitally with scanned slides. To investigate the difference between white light microscopy and digitally scanned slides, one pathologist assessed all the samples a second time on the other modality after an interval of 3 months. All samples were evaluated with three different histological indices (Robarts Histopathological Index (RHI), Geboes Score (GS) and Nancy Index (NI)). All pathologists had a scoring protocol consisting of the three original publications of the indices, together with one aid article for Nancy Index and a miniature scoring atlas produced by the developers of RHI.

3.3 Statistical methods

In paper 1 the difference in gene expression between the groups was investigated using Two-way ANCOVA models. Assumption of normality was checked with histograms, Q-Q plots and the Shapiro-Wilks test. Genes not displaying a normal distribution were evaluated with appropriate non-parametric tests such as Mann-Whitney U-test. All tests were two-sided and p-values below 0.05 were considered statistically significant. Benjamini-Hochberg correction for multiple comparisons was calculated and presented. The hydrolysis probe dataset was larger and investigated the difference between two groups (UC remission and non-IBD). The final model could adjust for age, gender, MES and GS. The SYBR-green dataset was smaller and investigated differences between three groups (non-IBD, UC remission and UC active). Therefore, this model could be adjusted for age and gender only.

The assessment of the reliability of the histologic evaluation in paper 2, was performed with two different methods. First by calculation of the Intraclass Correlation Coefficient (ICC) and secondly by a visual representation with Bland-Altman plots. The inter-rater ICC was calculated with two-way random, average score ICC for consistency (C,3), while the intra-rater ICC was calculated as a single score ICC for absolute agreement (A,1). The Bland-Altman plot is usually performed between two variables, but Jones et al. developed a method to visualize agreement between multiple raters (220). In this method each rater's value for a sample is plotted against the mean of all raters for that sample. To

facilitate comparison between the scores, all scores were standardized by dividing them on their theoretical max. Because of skewness in the raw data, all scores were transformed by square root.

Fleiss' kappa was applied on agreement evaluation between categorical variables and evaluated according to the definitions of Landis et al. (221). Systematic difference in rating between the raters was investigated with Kruskal-Wallis rank sum test. If significant, a sub-analysis with Wilcoxon rank sum test was performed to identify how the graders differed. Relationships between two dichotomous variables was assessed with Fisher exact test.

Variable selection was a challenge in paper 3 as there was a high number of covariates and a low number of cases. In total there were 42 covariates, 41 cases and 14 events. There are several ways of dealing with this, such as multiple testing and adjusted p-values, stepwise regression, or penalized regression. In multiple testing, all variables are run through a univariate regression analysis and the variables are selected according to a p-value adjusted for multiple comparison. The problem with this approach is that it does not account for effect size. Stepwise regression is challenged both by a high number of covariates compared to cases and multi-collinearity (222). Penalized regression handles these issues better. In penalized regression, a penalizing factor is added to the regression model to adjust for having too many variables. The least absolute shrinkage and selection operator (LASSO) method has the effect of reducing the coefficients of variables with minor contribution to the model to almost zero. This qualifies it to make a subset selection of variables. Finding the optimal penalizing factor is often done through cross validation, and the glmnet package for R can calculate LASSO regression with cross validation. Due to the “random” partition of the data set for the cross validation there is an element of non-random “randomness”. To account for this, the LASSO regression and cross validation were performed 10 000 times with different seeds, and the number of covariates with non-zero coefficients were counted. This method was proposed by Vinvand in a master thesis as a way to deal with preselection bias in high dimensional data (223).

4 Summary of results

4.1 Paper 1 - Mucosal transcript characterization of Ulcerative colitis in clinical remission

"The mucosa of UC in clinical remission differs from normal mucosa, suggesting a dysregulation of inflammatory and wound healing mechanisms"

72 participants (44 UC remission, 4 UC active and 24 non-IBD) donated biological material for transcriptional analysis. The aim was to compare known inflammatory and healing mediators in the mucosa of UC in clinical remission with normal controls.

Among the 51 mucosal transcripts examined, ten were significantly regulated between UC remission and non-IBD., eight were upregulated (*IL1B, IL33, TNF, TRAF1, CLDN2, STAT1, STAT3* and *IL13Ra2*) and two were downregulated (*TBX21* and *TGFB1*). Between UC active and non-IBD nine transcripts were significantly upregulated (*ADAM17*, *CASP8, TRAF1, CLDN2, DEFB1, IL13RA2, STAT1, STAT3* and *TFF3*) and one downregulated (*CHUK*). *IL1B* differed significantly in expression between genders, where males had a higher expression than females. All comparisons were adjusted for age and gender

Mayo Endoscopic Score (MES) of 1 differed from 0 on a transcript level, as several master transcription factors for T-cell development (*TBX21, GATA3, RORC, SPI1* and *FORXP3*) were upregulated in MES 1. In addition, *IL6, IL10, IL33*, *ST2, TLR4* and *TGFB1* were upregulated in MES 1.

4.2 Paper 2 - Real life evaluation of histologic scores for Ulcerative Colitis in remission

"A substantial amount of UC patients in clinical and endoscopic remission display inflammation on a histological level, but the ability to classify these patients accurately and consistently could be improved."

Mucosal biopsies from 41 UC patients in clinical remission were collected for histologic evaluation by three pathologists according to three different indices.

The Inter Class Correlation (ICC) coefficient for Geboes Score (GS), Robarts Histopathological Index (RHI) and Nancy Index (NI) were 0.85, 0.73 and 0.70, respectively, and the limits of agreement were ±6.1, ±4.0 and ±1.4. One pathologist rated systematically higher than the other two on all three indices. Neither colon location nor medication seemed to have any association with the histological scores. Categorical agreement on the remission state was assessed with Kappa Fleiss and showed a fair to moderate agreement between the pathologists.

Mayo endoscopic subgrade and Ulcerative Colitis clinical score did not show association with any of the histological scores. Despite clinical and endoscopic remission, 7-35% of the patients displayed histological inflammation on a level classified as active disease, depending on index and cut off.

4.3 Paper 3 - IFNG:IL33 ratio predict relapse in UC remission patients

"**A ratio between IFNG and IL33 is predictive of relapse and could be a potential new biomarker for relapse in UC remission patient"**

42 variables were gathered form 41 participants in clinical and endoscopic remission of UC in order to investigate their predictive abilities of relapse.

Among the 41 participants, 14 experienced relapses during the follow-up. The median follow-up time was 6.5 months (IQR 6.6) for those who experienced relapse and 12 months (IQR 11) for the non-relapsers. Of 42 variables eight showed non-zero coefficients in a LASSO regression analysis. Of these eight the best performing factor was a ratio between IFNG and IL33. The univariate cox regression analysis showed 5.3 times (95%Cl = 1.8-15.4) higher risk of relapse in a low IFNG:IL33 ratio group than in a high group. The IFNG:IL33 ratio performed better than both UCCS and MES in predicting relapse. Histologic evaluation could not predict relapse. Immunostaining showed IL33 presence in endothelial cells and mononuclear cells in the lamina propria. No difference in IL33 distribution or amount between UC remission and non-IBD was observed.

5 Discussion

5.1 Methodological considerations

5.1.1 Study design

An overview of the different study designs is presented in table 4. The case-control design in paper 1 restricted the possibilities of concluding the direction of the found association. Paper 2 is an observational study of the reliability of histologic evaluation. It does not address whether a pathologist evaluated true or false, but rather how similar three pathologists rated UC remission biopsies. The design of paper 3 made it possible to evaluate the predictive impact of the variables evaluated. Their predictive capabilities, however, need to be validated in another independent data set.

5.1.2 Internal validity

Internal validity is defined as the extent to which the observed results represent the truth in the studied population and not methodological errors. The errors may be divided into selection bias, information bias and confounders. In the following chapters the methodological challenges of the main analytical methods used in this thesis will be debated. The methods applied were quantitative polymerase chain reaction (qPCR) and histology.

5.1.2.1 Selection bias

Selection bias may arise when the subjects included in the study differ from the source of the population. In paper 1 there could be a potential for selection bias due to the selection of non-IBD controls. The patients were referred due to GI-symptoms or suspicion of GI-cancer, which resulted in an age difference between the case and control group. The mean age for the UC group was 40.5 (sd: 12.9) years, compared to 54.6 (sd:18.6) years for the non-IBD, which was unfortunate but difficult to avoid. To adjust the for the age difference, age ought to be included in a multivariate analysis. Ideally, the controls should have been selected from a healthy population without complaints, but this was difficult due to the nature of the sample collection procedure. By only including non-IBD patients having no irritable bowel syndrome (IBS) symptoms, no polyps in rectum lager than 5 mm and 0 on both the MES and the Geboes histological scores, the control group approximated a healthy background population as much as possible.

Patients declining to enter the study might also result in selection bias as they could be systematically different from those who were included. In this study the overwhelming majority accepted participation and the effect size of this bias would be minute.

5.1.2.2 Information bias

Information bias is a systematic difference from the truth that arises at the collection, recall, recording, and handling of information in a study, including how missing data is dealt with (224). Information bias will be discussed in relation to qPCR, histology, assessment of disease activity and endpoint registry.

Limitation of transcript analysis

The qPCR analysis provides a snapshot of the amount of mRNA in that exact location at that time. Wu et al. found that a single pinch biopsy from the colon was highly reflective of that segment (225). Nevertheless, mRNA has a relatively short half-life and there are many post-translational factors

determining whether the templates result in a functional protein (226). Ultimately, the proteins are the direct executors of life processes, and any qPCR experiment should be validated on a protein level. This can be done by immunohistochemistry or mass spectrometry. These methods are either more time-consuming or more expensive, and qPCR serves well in a discovery phase as a hypothesis generator. The key assumption is that no proteins exist without a previous mRNA template and, to a certain degree, that there is a correlation between the amounts of transcript and protein (227, 228).

A limitation of SYBR-green assays is the sensitivity for genomic DNA (gDNA). As SYBR-green binds unspecific to double stranded DNA, any failure in completely removing gDNA or by contamination could produce an erroneous result. The length of a standard amplicon should be 50-200 base pairs (bp) while gDNA usually is much longer due to introns (229). A longer length will allow many SYBR-green fluorophore to bind and even minor amounts of gDNA would produce a strong signal. Proper handling limited the likelihood of this to happen and post-PCR melting point analysis allows detection of any false signals

Gene selection

The genes of interest (GOI) were selected based on literature and previous assays present in our lab. They do not give a full picture of all the different transcribed genes in remission but function rather as a targeted investigation based on previous knowledge. The selected genes were found to be important in animal models but unproven in human studies or genes found to be central in active UC but uncertain in remission. Some genes were representatives of the cell population and gave an indication of cell composition in the mucosa.

Clinical and endoscopic factors

Remission was defined as Mayo clinical score/UCCS of 0 or 1, with an endoscopic subscore of 0 or 1. No points were allowed on rectal bleeding and the total Mayo score could not be larger than 1. This is widely applied definition (134, 156).

The factors MES and UCCS in paper 1 and 3 were partly subjective interpretations and consequently there was a risk for misclassification. The main purpose of paper 2 was to address problems with subjective evaluation, but with focus on histopathology. All three papers relied on patients classified as remission patients. As this was done by two partly subjective scores, misclassification of case and controls could be a possibility. In paper 1 such a misclassification could have resulted in some of the remission patients ending up the active group and vice versa. In order to have an effect on the results, the misclassification had to be skewed in one direction. For example, the physicians should have systematically underestimated or overestimated the patient's symptoms. There was, however, no indication that this was the case. The differences found between MES 0 and 1 could have been a result of different operators making the assessment, as the untrained inter-rater agreement is moderate (230). In paper 2 we acknowledged the potential information bias in evaluation of histologic samples. Evaluating the intra class correlation and kappa Fleiss gave an understanding of the extent of this issue before investigating if histology might predict relapse in paper 3.

Relapse

In paper 3 the factors from papers 1 and 2 were used to investigate if they could predict relapse in this sample population. Relapse was defined as any increase in medication and contact with health care providers. There was a potential for information bias as some patients might have had a relapse

without contacting the health care provider. This would have resulted in fewer relapses in the data set than the true value. The same result could be true if a patient moved to another hospital during the follow up time. The relapse rate, however, was 34% with a median follow-up time of 8 months which was in line with previous reports (146).

Confounding

A confounding variable is a third unmeasured variable that influences both the independent and the dependent variable resulting in an erroneous understanding of the association between the two measured variables. Age, sex, and disease duration were variables likely to influence the gene expression in the colon and should be included in the analyses. In paper 1 the hydrolysis probe results were analysed with a model that adjusted for sex, age, MES and GS. Unfortunately, due to a lack of power the SYBR-green assay results could only be adjusted for age and sex. In paper 3 the lack of power prevented any adjusting for potential confounding variables. This proved to not be detrimental as all variables were run through the LASSO regression and only age and disease duration were potentially important. Regardless, with such small coefficients the real impact would still have been negligible. Furthermore, the associations reported in paper 3 need to be validated in an independent larger dataset where the potential confounding factors could be more thoroughly investigated.

In paper 2 the agreement between three pathologists evaluating UC remission biopsies were assessed. A flaw in the study design was that the pathologists were blinded to the biopsy locations. It is an established fact that there is a higher number of eosinophils in the proximal colon than in the distal segments (231). Therefore, an assessment of "normal eosinophils" was dependent on location. Also, one pathologist evaluated the slides digitally after scanning, which lowered the ability to detect the red granules, characteristic for eosinophils. These two confounders contribute to the artificially high disagreement between the pathologists regarding to eosinophils.

5.1.3 External validity

External validity refers to how well a study's outcome relates to the general population in focus. Internal and external validities are often in opposition to each other, where one must make sacrifice on one end to improve the other. The inclusion criteria did not discriminate on medication and thereby improving the external validity as a wider spectrum of treatment regimes were represented in the material. Also, including patients in different clinical situations (treatment stops, 6-months follow-ups or routine cancer screenings) increased the external validity in paper 1 and 3. In paper 2 the external validity was related to the pathologists rather than to the UC patients. As paper 2 included both one GI-specialized and two general pathologists the results were more representative for pathologists in general, compared to if only GI-specialists had been used. Nevertheless, even within the general pathologist population there is a variation in the number of evaluated IBD samples which probably will affect the application of the histologic scoring indices. Therefore, generalization of the results in paper 2 to other hospitals should be done with caution.

5.2 Main results

In-depth discussion of the main results can be found in the respective papers 1-3. In the following sections the potential and limitation of histology in the context of UC categorization will be discussed. This will be followed up by a discussion about remission classification with focus on immune mediators as biomarkers.

5.2.1 Histologic evaluation of remission

Could histology be the next target in the treatment of UC? Histology is an established tool in the diagnosis of UC with characteristic goblet cell depletion and crypt abscess features, but it has not been proven with respect to treatment decision and routine evaluation. In recent years there has been an increased focus on histology as treatment target and criteria for remission. There have been two large systematic reviews regarding this topic during the last 10 years, one by Bryant et al. and one by Battat et al (179, 232). Bryant suggests in 2014 that histology can be predictive of relapse in UC, but that it suffers from lack of standardization and validation as too many different indices and cut-offs are used. Battat et al. harmonize this notion in their paper reviewing histology's role in clinical trials. In a newly published paper, The European Crohn's and Colitis Organisation (ECCO) addresses the lack of standardization by recommending the use of either RHI or NI, and recommending specific cut-off for remission (RHI<4 and NI=0) (233). Although a protocol for biopsy collection in the diagnosis of UC is recommended, no recommendations are offered for histologic sample collection during routine investigation.

In paper 2 the agreement between three pathologists was investigated. Two of them were general pathologists while the third was a GI-specialized pathologist. The aim was to evaluate how coherent these pathologists rated remission biopsies, and to investigate if the agreement differed between different histologic indices in order to discover potential limitations of using histology as a treatment target. The three most applied histologic indices (GS; RHI and NI) were investigated to determine which was the most preferable. The results showed that all three indices had similar agreement, but there was a clear difference between the pathologists. The GI specialized pathologist rated consistently higher in all the indices. Interestingly, there was no difference in the inter-quartile range suggesting that the pathologists rated consistently with their subjective understanding of the indices. It also indicated that sub-specialization might affect accuracy but not necessarily the precision. The agreement for categorization of the patients into remission or active groups was only fair to moderate for all indices. In a clearer term, the pathologists agreed on whether a patient was in remission or had active disease in 51-89% of the cases, dependent of score and cut-off.

Several different cut-offs have been used in previous reports, and agreements have differed in either extreme end of the indices. The present study investigated if there was an agreement difference between strict (GS <2A.1, RHI <4 and NI = 0) and relaxed (GS <3.1, RHI <5, NI <2) cut-offs (172, 173, 181, 234-240). The cut-offs were based on previous literature and the results showed that the strict cut-offs had a lower percentage of agreement.

The results showed lower rater agreement than the developers of the scores have reported (176, 177). This could be due to lack of training or experience of our pathologists. In both the original RHI and NI validation papers the evaluating pathologists were given extensive training (176, 177), while our pathologists only received a scoring aid protocol (se Method chapter). It could be questioned whether a protocol was sufficient to ensure proper rating, but arguably this setting was closer to a real-world setting where general pathologists will be asked by clinicians to use disease specific scores without receiving specific training. In a similar study Villanacci et al. investigated the real-world usefulness of histology in UC using two differently experienced pathologists (241). They were not given specialized training to improve coherent ratings, but they were both GI-pathologists who were familiar with the scoring systems. Accordingly, they reported a very high agreement. The use of GI-specialized

pathologists in scientific studies may be reasonable but it limits the generalization of their results since sub-specialization outside tertiary care centres is rare.

The limit of agreement (LOA) gives the interval of error for a repeated measurement. The error includes both systematic error and random error. The results from paper 2 suggested that the limits of agreement were ±6.1, ±1.4 and ±4.0 for GS, NI and RHI respectively. For easier comparison, the scores were standardized which gave the following values: 0.53, 0.59 and 0.35. RHI had slightly better LOA than the other two, but in absolute numbers the interval was substantial. This indicated that histology at this level was rather insensitive to changes as a large portion of the difference observed could be attributed to error rather than actual difference. This was unfortunate as the histologic evaluation of remission patients is an exercise in observing minor changes and the majority of patients will have few features of inflammation.

Jairath et al. found good correlation and response between histology and other clinical parameters such as the Total Mayo score, while Lobatón et al. found poor correlation between GS and MES (180, 183). The results from paper 2 did not show any association between UCCS, MES and any of the histologic indices. This discrepancy in results might be attributed to inclusion differences. Jairath et al. used data from the Touchstone clinical trial which included participants representing the whole range of the histologic scores. Conversely, in paper 2 only patients in remission according to the latest guidelines were included. These strict inclusion criteria and long remission times for many of the participants reduced the number of samples with residual histologic inflammation. This revealed the true usefulness of histology since there were few participants in the higher end of the indices with obvious signs of inflammation. The strict selection might be interpreted as a “stress test” of the histological indices.

The substantial inter-rater variation limited the applicability of the histologic evaluation of UC patients in remission. A way to amend this could be by adopting a central reader approach. Despite performing the rating in two different manners, with white light microscopy and digitally on a screen with scanned slides, the intra-rater value was good to excellent, with exception for the eosinophile feature in GS. This indicated that little information was lost due to scanning and digitalizing the samples and it would be beneficial to have a specialized central reader evaluating the samples digitally. This could result in more coherent scoring, lower LOA and improved usefulness as there is likely to be improved resolution in the lower ends of the scores.

In conclusion, the recommendation of histology as a requirement for remission is still questionable, especially outside centers with sub-specialized GI-pathologists. Rather than having histology as a hard treatment target it would be more appropriately used as a soft aid in clinical decision.

5.2.2 Immunological remission profile

Gene expression is the foundation for all biological function in our organisms. Consequently, investigating the mucosal gene expression in UC may potentially reveal the crux of the disease. Much focus has been aimed at the gene expression of UC in the inflammatory state and less on the remission state, especially in human samples (225, 242, 243). The inherent problem with evaluating active UC is the distinction between what is core UC pathology and what is a result of inflammation in general. By investigating the UC remission mucosa, one could get information un-distorted by inflammation. This could hold the key to unravel the UC pathogenesis. As mentioned in the method section, there may be

a difference between gene expression and functional protein. Therefore, any findings need to be validated on protein level.

In paper 1, the immunological profile in the mucosa of UC patients in remission was investigated and compared to non-IBD controls and active UC. The transcripts were analysed either with hydrolysis probe or SYBR-Green. The aim was to characterize if, and how, the UC remission mucosa differed from the non-IBD in a search for knowledge about the core UC pathology. A total 51 genes were tested and 15 of these were differently expressed between the groups investigated. *TNF*, *IL33*, *IL1B, TRAF1, CLDN2, IL13RA2, STAT1* and *STAT3* were upregulated in UC remission compared to non-IBD. *TBX21* and *TGFB1* were downregulated in the same comparison. *ADAM17, CASP8, TRAF1, CLDN2, DEFB1, IL13RA2, STAT1, STAT3* and *TFF3* were upregulated in active UC compared controls. *CHUK* was the sole transcript to be down regulated in active UC.

TNF and IL1B are primarily pro-inflammatory, indicating that there is residual inflammatory signalling in UC remission mucosa on a transcriptional level independent of both endoscopic and histologic appearance. Interestingly, none of these genes correlated with histology (unpublished data). The role of IL33 in this setting was difficult to ascertain as it has dualistic properties. Immunohistochemistry did not reveal any difference in IL33 amount when comparing UC remission to non-IBD participants (unpublished data). The distribution of IL33 in the mucosa was also similar between the two groups, and it was mainly found in endothelial and mononuclear cells in the lamina propria. Gundersen et al. found IL33 to be located in the epithelial cells of active UC (111). The different localization of IL33 in our findings might suggest that it was exerting its anti-inflammatory properties.

TGFB1 is an anti-inflammatory cytokine and in the results from paper 1 it was found to be downregulated in remission compared to non-IBD. TGFB1 is important for immune tolerance as an inducer of Tregs and in a quiescent state the tolerogenic processes should be increased, or on par, in comparison with non-IBD. TGFB1 is also found to be important in maintaining the intestinal barrier in animal models(120). The reduced expression could be a factor in the relapsing nature of UC as reduced TGFB1 could result in both weakened barrier and reduced tolerance function, which consequently would result in an active immune response. Other reports on *TGFB1* in UC mucosa are conflicting (118, 244).

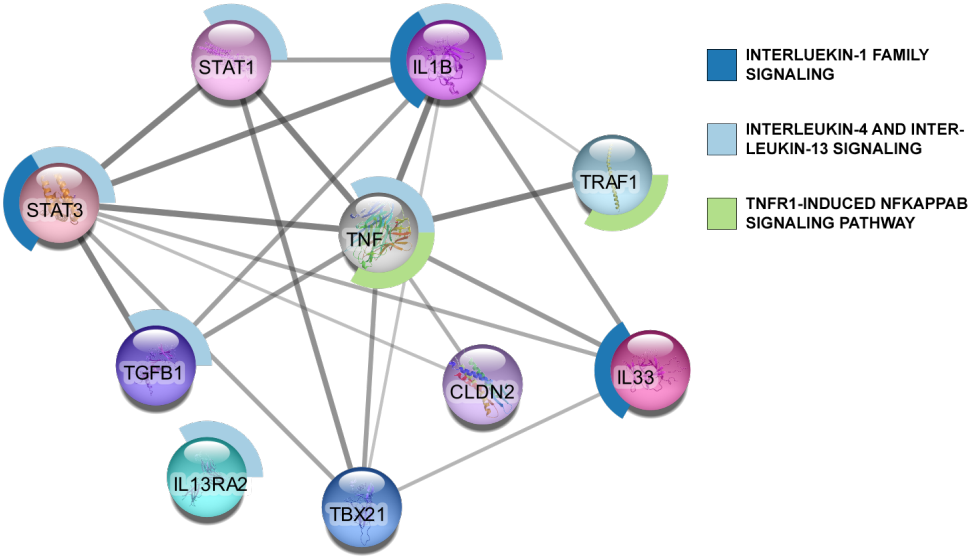
IL1B, TGFB1 and IL13RA2 are implicated in wound healing and pro-fibrotic processes (97). TGFB1 and IL1B regulate the production extracellular matrix in fibroblasts (245). Interestingly, *IL1B* and *TGFB1* were inversely expressed which challenged the understanding. A study investigating fibrotic mediators in UC suggests that the balance of TGFB isoforms determines their functions (96). Unfortunately, the other isoforms were not investigated in this study. *IL13RA2* was found to be upregulated in both UC active and UC remission. This might reflect either, the profibrotic properties or the proinflammatory properties IL13RA2 is found to have in animal models (246, 247). Information on IL13RA2 in human studies is scarce and it is difficult to accurately determine its function without further investigation. However, with increased expression of *IL13RA2* and *IL1B,* it could point to ongoing wound healing/fibrotic processes.

In paper 1, TRAF1 was found to be upregulated in remission mucosa. It is thought to function as a decoy receptor for TNF, which could counteract the effect of the increased TNF in the mucosa (248).

Interestingly, another upregulated gene in the remission group was *CLDN2*. CLDN2 is a protein which regulates tight-junctions in the epithelium, and an increase in the protein is associated to barrier dysfunction. *CLDN2* is found to be upregulated in UC and to correlate with disease severity, but it is not previously described to be upregulated in remission mucosa (249, 250). The upregulated expression of *CLDN2* in remission mucosa was supportive of the "leaky gut" hypothesis.

To evaluate these transcripts in a bigger perspective, a functional enrichment analysis of the ten remission transcripts was performed to evaluate which pathways and what interactions were present. Figure 3 shows the result of the analysis preformed with open-source Cytoscape software (251-254). The partial donut represents the top three reactome pathways these proteins are included in, evaluated by number of genes and lowest false discovery rate (255).

Figure 3 Functional Enrichment Analysis of UC Remission. PPI enrichment = 5.05E-9, indicating that there are more interactions between these proteins than would be expected for a random sample of proteins. The thickness of the line represents the strength of the evidence, including text-mining, co-expression and known interactions.



The network displays TNF node as a central agent in the cluster as most of the nodes have a strong relation to it. Both TNF and the IL1 family signalling are mediated through NF-κB pathway, which indicate that this pathway is active in remission. TNF and the NF-κB pathway are previously described as important in active UC (99, 256, 257). It was surprising that 6 of the 10 differently transcribed genes were implicated in the IL4 and IL13 signalling, as these are considered typical TH2 cytokines and neither *GATA3* (Th2 master transcription factor), *IL4*, nor *IL13* were differentially regulated. On the other hand, it could be argued that UC is an atypical TH2 mediated disease and this finding could be interpreted as a residual from an unresolved atypical TH2 mediated inflammation. As

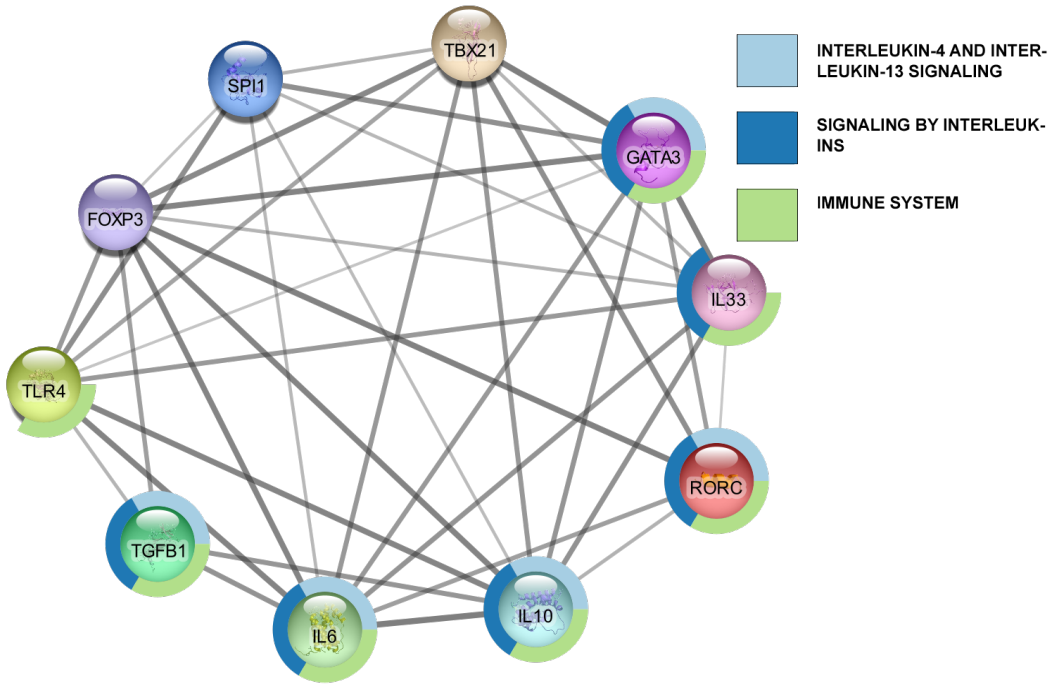
mentioned in the introduction, the partition of IBD into CD as a TH1 driven disease and UC as a TH2 driven is contentious.

Overall, the UC remission patients had an increase of pro-inflammatory transcripts associated to NF-κB pathway. In addition, mediators of the epithelium and barrier function were differently regulated in UC remission patients.

Investigating the expression difference between MES 1 and 0 was interesting due to the findings that MES 0 had better outcome than MES 1 (167, 168). Which genes were differently transcribed between the two levels? In paper 1, 11 genes were found to be upregulated in MES 1 compared to MES 0 and there was a mixture of proinflammatory (*TNF, IL6*) and anti-inflammatory (*TGFB, IL10*) cytokines. This increase in cytokines could mirror the minor inflammatory activity that was registered by endoscopy. Interestingly, several of the master transcription factors for the central T-cells in UC pathology were upregulated. TBX21, GATA3, SPI1, RORC and FOXP3 are the master transcription factors for TH1, TH2, TH9, TH17 and Treg. This could be a result of the increased cellularity in the mucosa, although this was not reflected histologically as none of these genes correlated with the histological score. Nevertheless, this result suggested that there was an increased T-cell differentiation activity in MES 1. In a similar study Fukaura et al. find no difference in gene expression between MES 0 and 1 regarding master transcription factors, and only IFNG differs between controls and UC remission (205).

The top three reactome pathways gathered from the functional enrichment analysis were IL4 and IL13 signalling and two unspecific immune pathways (Figure 4). Unlike the remission/normal analysis there was no apparent central node and the network was more integrated.

Figure 4 Functional Enrichment Analysis of MES Grade. PPI enrichment = 1.0E-16. The thickness of the line represents the strength of the evidence, this includes text-mining, co-expression and known interaction.



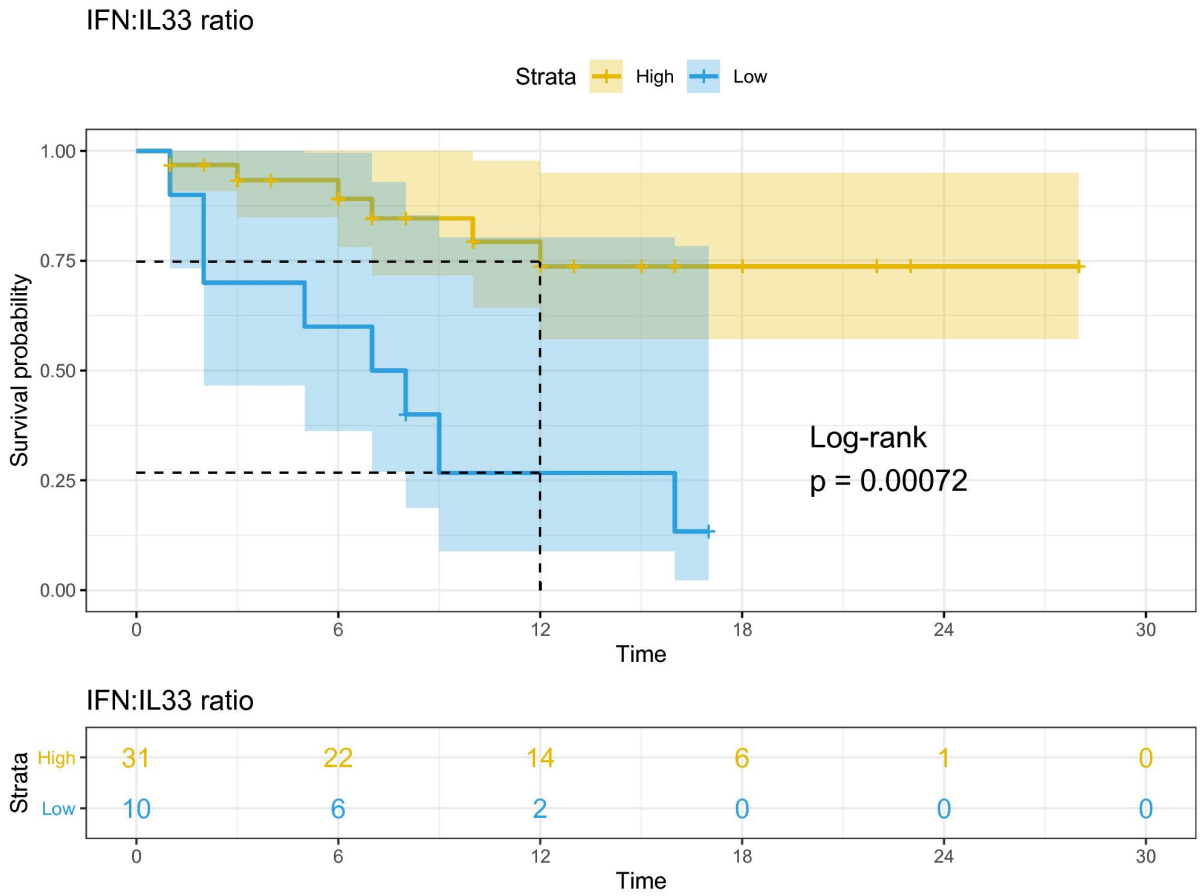
5.2.3 Relapse biomarkers

Remission evaluation has had the direction of continuously increasing its resolution to find smaller and smaller signs of inflammation. In the beginning the evaluation was solely based on symptoms. In the last decade endoscopy has been added as a part of standard evaluation, and now histology is on the cusp of being included. Transcriptional markers could come to play a part in this evaluation.

In paper 3, a regression analysis including 42 variables was made across clinical, endoscopic, histological and transcriptional factors to evaluate if one or more of them could predict relapse. The median follow-up time was 8 months and the median time to relapse was 6.5 months. 14 of the 41 included patients experienced relapse during the follow-up time. Of the 42 covariates analysed with LASSO regression, 8 covariates had non-zero coefficients and thus a potential predictive ability. Of these eight, three were excluded due to too low coefficients and the five remaining were assessed with a univariate regression model. *IL33, IL1B,* MES and UCCS had increased risk for relapse with higher levels, while *IFNG* displayed reduced risk with higher levels. Worth remembering is that a higher level i.e ΔC_T value, means less actual mRNA transcript in the sample. As there were transcripts with opposing effects two ratios were created, IFNG:IL1B and IFNG:IL33. Of these, IFNG:IL33 performed better with a higher odds ratio and p-value. The beneficial ratio category had relatively lower ΔC_T of *IL33* and higher of *IFNG.* An optimal cut-off value was found by ROC analysis and Youden index resulting in a specificity of 92.6% and a sensitivity of 57.1% with an AUC of 76.5. With this cut-off the PPV and NPV were 80% and 80.6%, respectively. The univariate cox analysis showed that patients in the low ratio category had 5.3 (95%Cl = 1.8-15.4) times increased risk of experiencing relapse. The Kaplan-Meier-plot (Figure 5) displayed a clear difference in relapse-free survival after

twelve months follow-up. About 25% in the low ratio category had not experienced a relapse compared to 75% in the high category group.

Figure 5 Survival plot of the IFNG:IL33 ratio. Kaplan-Meier plot illustrating the difference in survival probability between a high and low ratio of IFNG:IL33 and tested with a log-rank test. Dotted lines represent 12 months survival.



This ratio has not been previously described as a biomarker for relapse. The results indicated that in this remission setting, the presence of IL33 was protective. IL33 is described to be pleiotropic, and previous papers report it to be normalized in remission (111, 258). In paper 1, IL33 was found to be upregulated in UC compared to non-IBD and to be upregulated in MES 1 compared 0. The explanation for this was not clear, but likely, it reflected the dualistic properties and the location specific effects of IL33. The immunohistochemistry analysis showed IL33 expression in lamina propria mononuclear cells, which could be macrophages. Seo et al. found that IL33 switches M1 macrophages to the more tolerogenic M2 subtype. Therefore, in the context of remission and low to no inflammatory mediators present, reduced IL33 could result in a more pathogenic macrophage population. The finding that IFNG was pathogenic was in line with the current knowledge but it has not been described as a predictor of relapse (259, 260). IFNG and IL33 do not show any predictive abilities in another study (205).

Although MES came up as non-zero covariate it was not significant in a univariate model (Odd ratio 2.75, 95% CL:0.86-8.80). This was likely due to a power issue as MES has been described to be predictive of relapse in larger studies (167). Histology was evaluated as a continuous score, and categorically as active or in remission, but with two different cut-offs (Strict and Relaxed, see Paper 2). Interestingly, the histologic evaluation did not show ability to predict relapse, regardless of indices applied. Geboes remission category did come up as a non-zero variable but had an infinite coefficient and a high p-value. Histology has previously been reported to be predictive of relapse (180, 261). A probable explanation was the high variability in the histologic assessment, which could mask any potential predictive ability. With a central reader approach the results might have been different.

In summary low ratios of IFNG and IL33 were predictive of relapse, while MES and UCCS were limited by power issues and histology was not predictive of relapse.

6 Conclusion and implications

6.1 Conclusion

Aim 1: To characterize mucosal transcript of UC patients in clinical remission

- The remission mucosa was dominated by transcripts of pro-inflammatory mediators such as TNF and IL1B, while the anti-inflammatory cytokine TGFB1 was down regulated. The gene expression difference between the endoscopic grade 0 and 1 was dominated by the master transcription factors for several T-helper cells as well as by a mixture between pro-inflammatory and anti-inflammatory cytokines.

Aim 2: To evaluate the reliability of the three most applied histologic indices in patients with endoscopy defined remission.

- The reliability of histology was moderate for all the top three histologic indices and none of the indices were preferable with regard to test characteristics. The experience of the pathologist had a greater impact on reliability than the indices. Although there was a difference in accuracy between the pathologists the precision was similar.

Aim 3: Investigate if any clinical and histological factors and/or gene transcripts can predict relapse/remission..

- A ratio of IFNG and IL33 could predict relapse, while histology could not. Symptomatic, endoscopic and histologic evaluations did not show a significant ability to predict relapse. In the case of endoscopy, it could have been due to lack of power.

6.2 Clinical implication

UC is a severe chronic disease that is debilitating for the patient and is inflicting substantial health-care costs on society. Improved management by precision medicine will most likely benefit the patient by reducing the frequency of relapse through a more accurate use of medication. To achieve this, proper descriptions of the different states and treatment goals for the disease are needed.

Regarding histology's role in the remission definition, the present data could not warrant its inclusion. The current methods of evaluation were too prone to high variance resulting in an insensitive and inaccurate result. Histology could be used as an aid in the surveillance of the disease, as it could detect inflammation where endoscopy could not.

Precision medicine refers to the "tailoring of medical treatment to the individual characteristics of each patient" (262). There is evidence for biomarkers that can stratify patients according to the future course and outcome of disease (190, 263) and biomarkers that indicate safe de-escalation of treatment (202, 264). The next step in precision medicine will be to accurately assess the risk of imminent relapse in UC patients. The current surveillance algorithm includes symptoms, endoscopy, and faecal markers. In the future, transcriptional biomarkers might be included due to the disease-specific and objective nature of these markers. The feasibility of using mucosal markers is quite good as there is little additional work in taking an extra biopsy for transcript analysis during endoscopy.

These steps will take UC treatment closer to achieving a precise and accurate evaluation of the remission status. In a possible future, patients could receive tailored drugs and follow-up regimes based on specific risk factors, when diagnosed. Patients with certain factors could be given a more intensified follow-up program with regular endoscopy including mucosal transcript analysis to prevent relapse or neoplasia. For other patients, a more relaxed program might be appropriate, where quarterly faeces analysis could be sufficient.

6.3 Research implication

The immunological profile found in the present study was on transcriptional levels. To reveal the mechanisms and the wider implication of these findings it will be necessary to investigate associated genes as well as validating these findings on a protein level. Post-translational modification can change the protein expression in such a way that it would not reflect the transcript findings. Identification of the cell population expressing the cytokines in question, is also of interest. To put the pathology behind UC in a bigger picture, a "system biology" approach is necessary. In such an approach, data from different branches of biological research such as transcriptomics, proteomics, metabolomics and epigenomics are integrated. Combined with machine learning this could lead to investigating biology in unprecedented ways.

The predictors of relapse should be validated in a prospective study where patients in the low ratio group will have escalated treatment to prevent relapse. The ratio should also be investigated with a multi-omics approach in order to elucidate the cause of predictive abilities.

Histology has great potential as an adjuvant to clinical decision making but is limited by its subjective nature of the evaluation. To reduce the subjectivity, future research should focus on how to optimize a central reader approach, such as standardization of collection, preparation, and digitalization. Also, with these things in place, a new and promising possibility opens up in artificial intelligent image recognition (265).

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ORIGINAL ARTICLE

Mucosal gene transcription of ulcerative colitis in endoscopic remission

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ABSTRACT

Aim/Objective: Ulcerative colitis (UC) is a chronic inflammatory bowel disease. In UC, a wide range of criteria are used for disease remission, with few studies investigating the differences between disease remission and normal control groups. This paper compares known inflammatory and healing mediators in the mucosa of UC in clinical remission and normal controls, in order to better describe the remission state.

Method: Mucosal biopsies from 72 study participants (48 UC and 24 normal controls) were included from the Advanced Study of Inflammatory Bowel Disease (ASIB Study), Arctic University of Norway, Norway. Clinical remission was defined as Mayo clinical score $\leq$ 2, with endoscopic subscores of $\leq$ 1. Targeted gene transcription analyses were performed using hydrolysis probes and SYBR-green.

Results: Among the mucosal transcripts examined, 10 genes were regulated in remission versus normal controls, 8 upregulated pro-inflammatory transcripts (*IL1B*, *IL33*, *TNF*, *TRAF1*, *CLDN2*, *STAT1*, *STAT3* and *IL13Ra2*) and 2 downregulated (pro-inflammatory *TBX21* and anti-inflammatory *TGFB1*). In total, 14 transcripts were regulated between the investigated groups. Several master transcription factors for T-cell development were upregulated in patients with Mayo endoscopic score of 1 in comparison to 0.

Conclusions: The mucosa of UC in clinical and endoscopic remission differs from normal mucosa, suggesting a remaining dysregulation of inflammatory and wound healing mechanisms.

Abbreviations: IBD: Inflammatory bowel disease; UC: Ulcerative colitis; qPCR: Quantitative polymerase chain reaction; MIQE: Minimum information for publication of quantitative real-time PCR experiments; 5-ASA: 5-aminosalicylic acid; TNF: Tumor necrosis factor; mRNA: messenger ribonucleic acid; RNA: ribonucleic acid; DNA: Deoxyribonucleic acid; MES: Mayo endoscopic subscore; FC: Fold change; ECCO: European Crohn's and Colitis Organisation; RIN: RNA Integrity Number

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Introduction

Ulcerative colitis (UC) is a chronic relapsing inflammation of the colon. In the north European population, up to 0.5% are affected by UC, with a yearly healthcare cost estimated at 4.6–5.2 bn. Euros [1,2]. The etiology of the disease is not fully established but the four factors; genetic susceptibility, immune dysregulation, environmental factors and the gut microbiome are currently thought to play a central role in the pathogenesis of UC [3–5]. While only surgery is curative, immune-suppressive drugs have proved to be the most effective pharmacological treatment of the disease. In particular anti-tumour necrosis factor (TNF) therapy has shown to be crucial in treatment of severe cases [6,7].

The disease activity of UC is cyclical, which means treatment is given when the patient has flare-ups and stopped or de-escalated when the patient is in remission after short- or long-term maintenance treatment. The term 'disease remission' is widely used in UC, however, there is no consensus on what constitutes remission. The latest ECCO guidelines (2017) suggest a combination of clinical parameters (stool frequency $\leq$ 3/day with no bleeding) and no mucosal lesions by endoscopy [8]. The British Society of Gastroenterology's IBD guidelines define clinical remission as mayo score $\leq$2 and no individual score $\leq$ 1 [9]. The lack of consensus has given rise to many different terms such as mucosal healing, histological remission and deep remission. In clinical studies, the Mayo endoscopic grade is often used for determining remission, with scores of 0 and 1 both accepted as 'remission mucosa' [10–12]. Although, latest reviews suggests Mayo 0 as treatment target [13]. Unfortunately, there are no studies comparing this two-value score on a translational level and this represents a knowledge gap. Altogether, these issues make it challenging for the clinician to evaluate whether a patient is in disease remission or not.

By investigating the difference between normal and mucosa in clinical remission, without the distortion of inflammation, we can get a better understanding of immunological dysfunction in UC, with special emphasis on endoscopic Mayo score 0 versus 1. Therefore, the objective of this study

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Supplemental data for this article can be accessed here.

Table 1. Baseline characteristics in ulcerative colitis in clinical remission, active and in normal controls.

	Normal controls	UC Remission	UC Active
qPCR (SYBR-green)			
Number	10	9	4
Gender (M/F)	7/3	3/6	2/2
Age (mean)	56.9	42.6	27.2
Biopsy location	1/9/0*	3/4/2*	2/1/1*
Average endoscopic score	0	0	2.25
qPCR (hydrolysis probe)			
Number	24	44	
Gender (M/F)	16/8	19/25	
Age (mean)	54.5	40.5	
Biopsy location	2/21/1*	23/15/6*	
Average endoscopic score	0	0.25	

*Rectum/Sigmoid colon/Unknown.

was to describe the colonic mucosa of patients that are in clinical and endoscopic remission with a focus on cytokine expression and signaling.

Material and methods

This study is a part of the Advanced Study of Inflammatory Bowel (ASIB) prospective study at the University Hospital of Northern Norway, Tromsø. All study participants gave written, informed consent. The study and storage of biological material was approved of by the Regional Committee (REK Nord ID:2012/1349).

Study populations

Participants with UC according to established diagnostic definitions [8] were recruited from the ASIB study. An overview is presented in Table 1. For the remission group, we recruited primarily patients with moderate/severe disease who had been treated with anti-TNF. Inclusion criteria: age between 18 and 80, Mayo clinical score of 0 or 1, with endoscopic subscore of 0 or 1. No points were allowed on rectal bleeding feature and a total Mayo score larger than 1 was not included [8].

UC active inclusion criteria were: Total Mayo score above 2 and endoscopic subscore of 2 or above [14]. Endoscopic signs active UC and no inflammation of ileum.

A control group of non-IBD patients screened with colonoscopy for colorectal cancer or mild gastrointestinal symptoms were included. Criteria for healthy controls where no diarrhea or other irritable bowel symptoms, as well as a completely normal endoscopy, with no polyps in sigmoid and no hyperplastic polyps in rectum lager than 5 mm.

Gene transcription measurement

The gene analysis was performed as close to the MIQE guidelines as possible [15]. Two different qPCR methods were used (Hydrolysis probe and SYBR-green).

Biopsy preparation

Biopsy collection was done during routine colonoscopies and immediately immersed in RNAlater (Qiagen N.V, Venlo, the Netherlands) and kept in room temperature for at least 24 h prior to storage at -80 °C.

RNA preparation

The biopsy sizes were within the range of 3–10 mg. The sample was then homogenized in the MagNa lyser instrument (Roche Diagnostics, Etterstad, Norge) for 40 s at 6500 rpm. After the sample was disrupted and lysated it was centrifuged for 3 min at 13,000 rpm. Total RNA extraction was done with QiaCube and AllPrep DNA/RNA mini kit (Qiagen N.V, Venlo, the Netherlands) according to the AllPrep DNA/RNA mini protocol for animal cells and tissue. Total RNA samples were stored at −80 °C. Total RNA concentrations were measured with Qubit® 3.0 Fluorometer (Thermofischer, Waltham, Massachusetts, USA). The RIN values averaged 8.4 (SD 1.7) as measured by an Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, California, USA). cDNA synthesis was done with RT^2 First Strand Kit using 0.5ug of total RNA.

Reverse transcription

Reverse transcriptions for the hydrolysis probe assays were performed with QuantiNova Reverse Transcription Kit, while, the SYBR-green assays utilized RT2 First strand Kit according to manufacturer's instructions.

qPCR

Levels of mRNA for the selected genes, were determined by real-time quantitative polymerase chain reaction (qPCR) on a BioRad CFX connect 96-well thermal cycler (Bio-Rad Laboratories AB, Hercules, California, United States). The dual labeled hydrolysis probes (TaqMan) were done with the QuantiNova Probe RT-PCR Kit (Qiagen); and the SYBR-green assays were done with the RT2 Profiler kit(Qiagen), all according to the manufacturer's instructions.

Thermal cycler protocol

The plates had a positive, negative and genomic control. All plates were read at standardized threshold values. For the hydrolysis probe assays a 2-step protocol was used: Denaturation 95 °C for 2 min, then [95 °C/5 s and 60 °C/5 s] repeated 40 times. All genes were normalized to Beta Actin (*ACTB*).

For the SYBR-green assays, a 2-step protocol was used: denaturation at 95 °C for 10 min, then [95 °C/15 s and 60 °C/60 s] repeated 40 times. All genes were normalized to the geometric mean between *HPRT1* and *RPLP0* as recommended by NormFinder analysis [16].

Primer design

The primers and hydrolysis probes for the experiment were designed using Beacon Designer v8 (PREMIER Biosoft International, Palo Alto, USA). To ensure specificity for mRNA, all probes spanned exon splicing sites and all primers and probes were run through a BLAST search to ensure

Table 2. All tested genes by the analysis method.

Method	Cytokines	Transcription factors	Receptors	Reference genes	Others
qPCR hydrolysis probe	*IFN, TNF, TGFB1, IL1B, IL4, IL6, IL8, IL10, IL13, IL17a/f IL18, IL21, IL22, IL23, IL33,*	*TBX, GATA3, RORC, FOXP3, SPI1,*	*TLR4, IL1RL1*	*ACTB*	
PCR-array – SYBR-green	*TNFSF15, IL22, IL13, IL10, IL9,*	*SMAD7, CHUK, STAT1, SPI1, STAT3, SMAD3,*	*IL1R2, IL9R, IL13RA2, TRAF1,TNFRSF6B TNFRSF25*	*PPIA, RPLP0, HPRT1*	*OCLN, TFF3, ADAM17, PTK2, CASP8, CCR2, DEFB1, BCL2, CLDN2*

specificity for the mRNA sequence in question. The efficiency of all assays were measured by analysis of a dilution series from a biopsy extract (Table S1). Primers and probes were ordered from Eurogentec, (Kaneka Eurogentec S.A, Seraing, Belgium).

SYBR-green PCR array

The SYBR-green assays were prefabricated plates that were ordered from Qiagen with 26 genes picked by association to TNF, t-cell differentiation and barrier permeability. An additional 3 were selected as reference genes, where 2 were used.

Statistics

Statistics were performed using IBM SPSS Statistics 24 and R statistics version 3.4.3 and Rstudio Version 1.1.442. Assumption of normality was investigated with histograms, Q-Q plots and Shapiro–Wilks test. Two-way ANOVA models were used to compare groups. Genes that did not display normal distribution were evaluated with appropriate non-parametric tests. To investigate the difference between the groups in we did a linear model to find the coefficient between clinical status groups and then calculated fold change (FC $= 2^{-\Delta\Delta CT}$). Benjamini Hochberg correction for multiple comparisons was calculated. All tests were two-sided and *p*-values below .05 were considered significant. The adjusted model used in the hydrolysis probe data set was diagnosis (UC or normal) by gene, adjusted for gender, age, Geboes score and endoscopic score. The same method was applied to the SYBR-green dataset but because of power issues the model was reduced to only include clinical status, age and gender.

Results

Overview of the differently expressed genes

In total, 22 gene transcripts were analyzed using a hydrolysis probe (Table 2). Between UC remission and controls five of these genes, *TBX21, TNF, IL1B, TGFB* and *IL33* showed a significant difference (Figure 1). Twenty-nine genes transcripts were analyzed with SYBR-green (Table 2) assays on three groups (UC active, UC remission and controls). Between UC remission and controls five genes (*TRAF, CLDN2, IL13RA2, STAT1, STAT3*) were differently expressed (Figure 2). These five and an additional five were regulated between UC active and controls with the SYBR-green assays (*ADAM17, CASP8, CHUK, DEFB1, TFF3*; Figure 3). Overview of differently transcribed genes between clinical remission mucosa and controls in regard to gene relationship are shown in Table 3.

Hydrolysis probe assays: Difference in mucosal transcripts between clinical remission and normal controls

Patients in clinical remission had 3.2-fold higher transcription of *IL1B* than control ($p = .001$). *IL1B* also displayed a gender difference where males had a fold change of 5.3 compared to 2.2 in females. *IL33* and *TNF* were up-regulated in clinical remission compared to control; FC= 1.7 ($p = .02$), and 2.0 ($p = .01$), respectively. *TBX21* and *TGFB1* were less expressed in clinical remission patients, FC = 0.2 ($p < .001$) and FC= 0.7 ($p = .038$), respectively. Almost all genes were significantly associated with the endoscopic subscore, see Table S2. Benjamini–Hochberg correction for multiple comparison sets a *p*-value of $p < .01$.

SYBR-green assays: Mucosal transcripts differs between clinical remission, active disease and normal controls

Ten genes were significantly different when comparing active disease and control with the SYBR-green assays (Figure 3). Of these 10, five genes were still upregulated when remission mucosa was compared to control (Figure 2). The following genes were differentially expressed*: Adam17* ($p = .013$), *CASP8* ($p = .001$), CHUK ($p = .006$), *CLDN2* ($p = .016$), *DEFB1* ($p = .029$), *IL13RA2* ($p < .001$), *STAT1* ($p = .007$), *STAT3* ($p = .016$), *TFF3* ($p = .001$), *TRAF1* ($p = .001$), see Table 3. Benjamini–Hochberg correction for multiple comparison sets a *p*-value of .019. The interaction term between gender and clinical status was significant for *STAT1*, *CLDN2* and *TRAF1*.

Difference in subscore Mayo 1 and 0

When comparing the clinical endoscopic Mayo score of 1 and 0 we found several genes that were differentially transcribed. Following adjustment for age and gender, 11 genes were found significantly up-regulated in Mayo endoscopic subscore 1 compared to subscore 0 (Figure 4).

Discussion

The main findings of the present study are 1: We found 10 differentially transcribed genes between patients in clinical remission and subjects with normal mucosa. Of these 10 genes, eight pro-inflammatory were up-regulated and two (pro-inflammatory *TBX21* and anti-inflammatory *TGFB1*) were

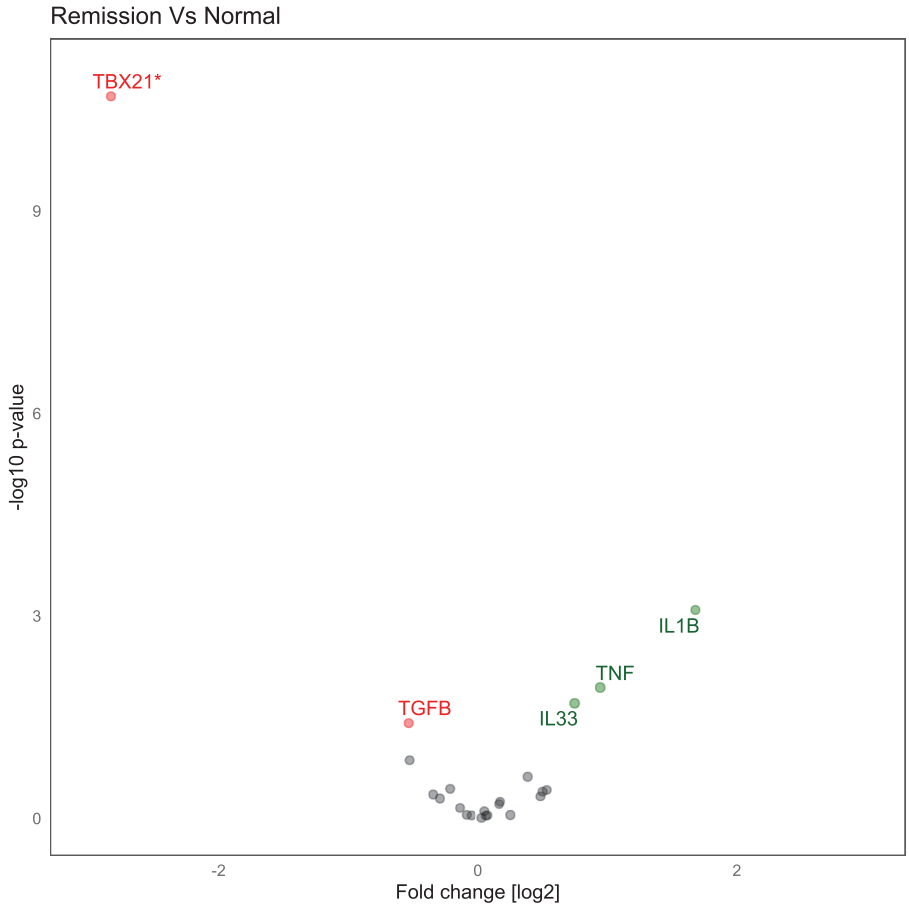

Figure 1. Volcano plot demonstrating differentially regulated genes between clinical remission and normal controls when adjusted for age, gender, endoscopic score and Geboes score. *TBX21 is analyzed with a nonparametric method (Mann–Whitney U-test). Genes analyzed with hydrolysis probe. All named genes are significant (<.05) and genes on the left-side are down-regulated, conversely, genes on the right-side are up-regulated.

down-regulated (Figures 1 and 2). 2: In addition, we found several T-cell transcription factors to be up-regulated in Mayo subscore 1 in comparison with 0 (Figure 4). The difference in transcription was small for most of the tested genes, this could be explained by the lack of active inflammation that could distort the results. As expected, the pro-inflammatory genes were up-regulated in active UC and the inhibitory genes, such as *CHUK*, were down-regulated.

The transcriptional difference between Mayo subscore 0 and 1

Our results show a difference in transcription between the Mayo endoscopic score (MES) of 0 and 1. Previous papers have shown that a MES of 0 gives a favorable outcome in relation to clinical remission rates [17–19]. All genes that were differentially transcribed were up-regulated in MES 1. Interestingly, most of the up-regulated genes were transcription factors for T-cell differentiation: *TBX21, GATA3, SPI1, RORC, FOXP3* that are central transcription factors for TH1, TH2, TH9, TH17, and Treg, respectively. This finding indicates that the t-cell differentiation of these linages of are still active in the Mayo subscore 1 score. Worth noticing is that *TBX21* is down-regulated in the remission mucosa compared to normal mucosa, but up-regulated in the MES 1 compared to MES 0. This could be because of medication suppressing t-cell development and the slightly increased cellularity one expects to find in mildly inflamed mucosa. The up-regulation the anti-inflammatory cytokines *TGFB* and *IL10* however suggest a counter-balanced inflammatory response. This is supported by the lack of *TNF* expression in MES 1. Still, it is important to notice that *TGFB* for the two groups (Mayo 0 and 1) as a whole was less expressed than in the normal group. To our knowledge this is the first investigation of gene transcript difference between Mayo subscores 0 and 1. This may have clinical implications for determining when to de-escalate treatment in UC in clinical remission. However, further investigation is warranted.

TNFR1/NF-Kb pathway

The results show that *TRAF1* has increased expression in both clinical remission and active UC mucosa, when compared to normal mucosa. This finding can be interpreted as an attempt to ameliorate the inflammation and reduce NFKB signaling. TRAF1 is a TNF receptor regulatory protein and is related to cell apoptosis/necroptosis and inflammation. It is suggested that the ratio between TRAF1 and TRAF2 is important for the effect TNF has on T-cell expansion [20]. Where TRAF1 is a negative regulator and TRAF2 is positive. This was confirmed in a study of TRAF1 deficient mice, where an increased response to TNF and

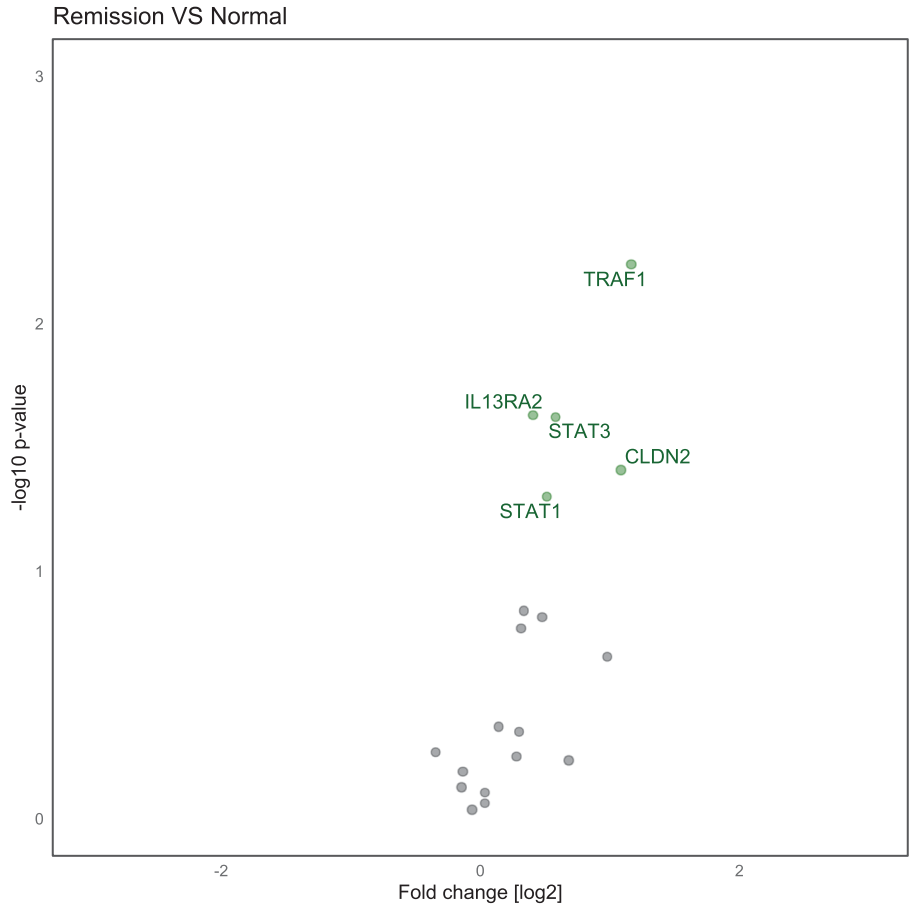

Figure 2. Volcano plot demonstrating differentially regulated genes when comparing Ulcerative colitis in clinical remission to normal controls. Genes are analyzed with SYBR-green and are adjusted for age and gender. Genes on the left-side are down-regulated and genes on the right-side are up-regulated.

higher T-cell proliferation was shown [21]. It is also suggested that TRAF1 has opposite actions depending on cleavage by CASP8. A full length TRAF1 is pro-cell survival, whereas a cleaved TRAF1 is pro-apoptotic when in a stimulated TNFR1 context [22]. Our findings are in line with other reports that TRAF1 is up-regulated in active UC and inhibit NFKB signaling [23].

Our results suggest that *CASP8* is up-regulated in active UC and not in clinical remission. CASP8 regulates apoptosis/necroptosis and is inhibited by NFKB activation. Our result is surprising as one would expect prolonged immune cell life to be beneficial in order to deal with infection. On the other hand, it could be a result of an attempt to down-regulate the inflammation. Up-regulation of TRAF1 could give a higher activation of CASP8 as TRAF1 regulates NFKB activation and may thereby remove inhibition for CASP8 activation, as mentioned earlier. Previous papers have reported no difference in CASP8 between control and UC [24].

The results of our analysis show that even in non-symptomatic and non-inflamed mucosa *TNF* is still up-regulated. TNF is one of the central cytokines in inflammation and acts as a pro-inflammatory cytokine. The up-regulation of TNF in clinical remission UC patients could be due to the cyclic nature of inflammation. Previous results are conflicting on the presence of TNF in remission mucosa [25,26]. This could be a result of different definitions and lack of consensus on what constitutes remission.

JAK-STAT pathway

Our study showed that *STAT1* and *STAT3* are up-regulated both in clinical remission and active UC when compared with normal mucosa, in line with previously published data [27,28]. STAT1 and STAT3 are a part of the JAK-STAT pathway which is responsible for several immunological functions and responses. Interestingly, our findings suggest that these signaling pathways are not just up-regulated in active inflammation, but also in clinical remission. What their functions are in clinical remission mucosa is difficult to say as they are involved in both pro- and anti-inflammatory signaling pathways dependent on cell type and substrate. Further eliciting their role in non-inflamed UC mucosa requires further studies. STAT3-mediated activation of acquired immune responses plays a pathogenic role in colitis by enhancing survival of T cells and by inducing TNF. In contrast, STAT3-mediated activation of innate responses contributes to the suppression of colitis by enhancing the mucosal repair and by inducing mucin production [29]. In either case, therapeutic targeting of the JAK-STAT signaling pathway with tofacitinib shows promising results [30].

Innate immune system

We found that *ADAM17* was up-regulated in active UC which is in keeping with previous research [31]. ADAM17 has been shown to cleave TNF to soluble TNF and can therefore be

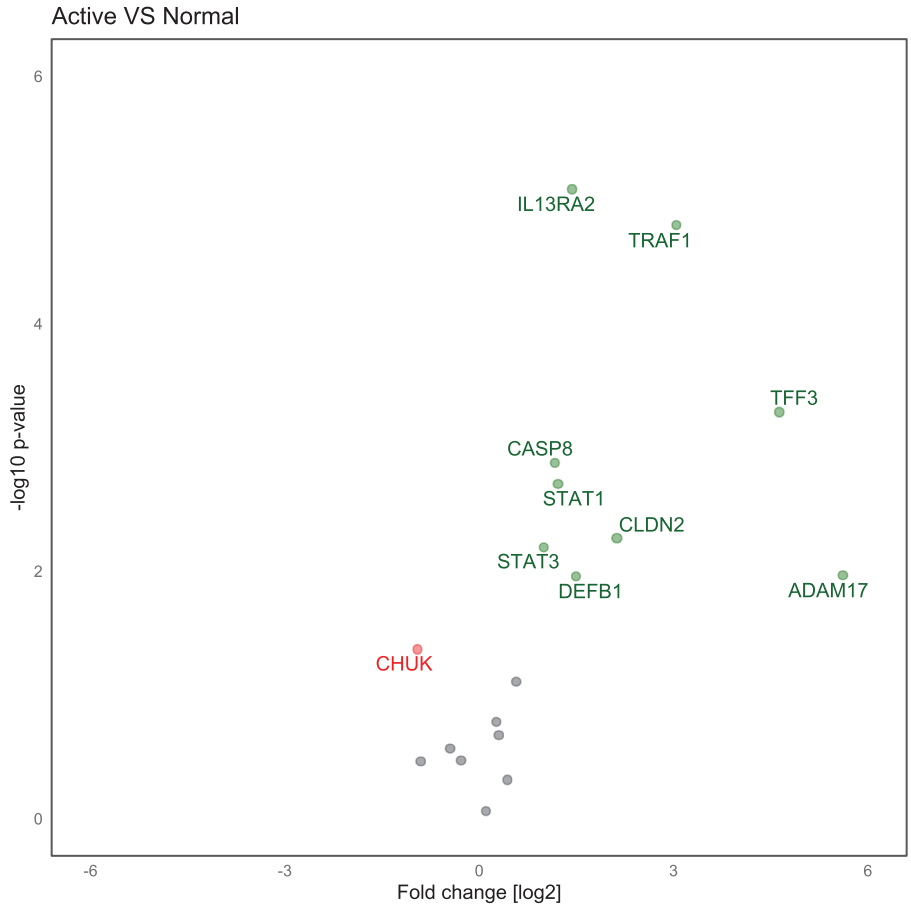

Figure 3. Volcano plot demonstrating differentially regulated genes when comparing active Ulcerative colitis to normal controls. Genes are analyzed with SYBR-green and are adjusted for age and gender. Genes on the left-side are down-regulated and genes on the right-side are up-regulated.

Table 3. Hydrolysis probes results are adjusted for age, gender, endoscopic subscore and Geboes score. SYBR-green results are adjusted for age and gender.

Gene	Remission vs. Normal	*p*-value	Mayo 0 vs. 1	*p*-value
Hydrolysis probe				
IL1b	1.6↑	<.001		Ns
TNF	0.9↑	.011		Ns
IL33	0.7↑	.019	0.8↑	.012
TGFb	0.5↓	.038	0.9↑	.000
TBX21	2.8↓	<.001	3.4↑	.000
IL6		Ns	1.2↑	.033
IL10		Ns	0.9↑	.010
TLR4		Ns	0.8↑	.001
IL1RL1		Ns	1.2↑	.000
SPI1		Ns	1.0↑	.004
FOXP3		Ns	1.6↑	.001
GATA3		Ns	1.4↑	.000
RORC		Ns	0.7↑	.039
SYBR-green	Remission vs. Normal	*p*-value	Active vs. Normal	*p*-value
Adam17		Ns	49↑	.011
CASP8		Ns	2.2↑	.001
TRAF1	2.2↑	.006	8,2↑	.001
CHUK		Ns	1.9↓	.043
CLDN2	2.12↑	.039	4.3↑	.005
DEFB1		Ns	2.8↑	.011
IL13RA2	1.32↑	.023	2.6↑	.001
STAT1	1.42↑	.049	2.3↑	.002
STAT3	1.49↑	.024	1.9↑	.006
TFF3		Ns	29↑	.001

Up-regulated genes are labelled ↑ while down-regulated genes are labelled ↓. For further details see text (Section Results) and Figures 1–3.

pro-inflammatory, on the other hand its role in activation of Erb-B ligands and Notch-1 pathway makes it a contributor to epithelial regeneration [32]. In addition, ADAM17 cleaves the IL1B decoy receptor IL1R2 into soluble sIL1R2 which is suggested to have anti-inflammatory effect [33,34]. We could not detect significant expression of *IL1R2* in any of our groups. Nevertheless, ADAM17 is a key protein in the role of TNF effects and plays an important role in inflammatory diseases.

IL1B is known to have a pro-inflammatory function in UC [35,36], and interestingly, the results show that *IL1B* was up-regulated in clinical remission mucosa, indicating a subclinical inflammation which could contribute to the cyclical nature of the disease.

Our results show that *IL33* was still up-regulated in clinical remission patients, although its receptor *IL1RL1* was not. The role of IL33 in UC is not clearly defined and it is likely dependent on the stage of inflammation [37,38]. IL33 has been implicated in intestinal fibrosis and in mucosal healing and goblet cell restoration [39–41]. Thus, the presence of *IL33* in UC clinical remission may represent ongoing wound healing/fibrogenesis and not an inflammatory process.

Our findings suggest that *TFF3* is expressed more in active inflammation. TFF3 is a protein secreted to the lumen from goblet cells in the colon and has a role in protection and

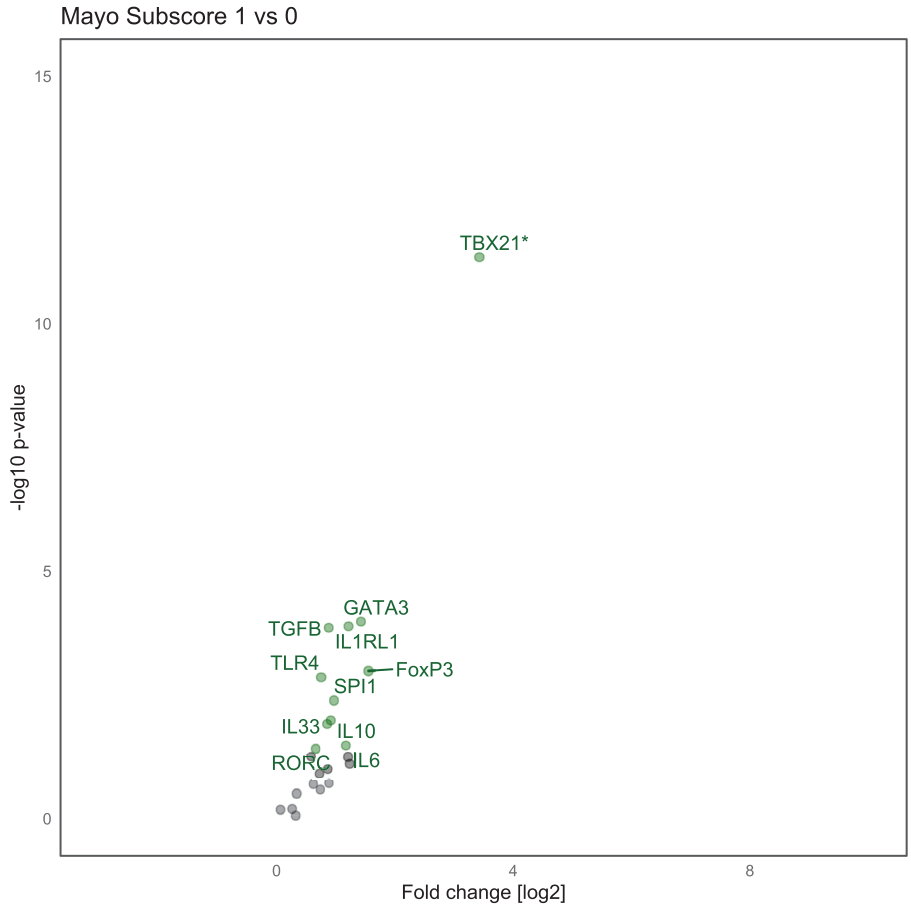

Figure 4. Volcano plot demonstrating differently translated genes between mayo endoscopic score 0 and 1. Analyzed with hydrolysis probe. Several transcription factors for T-cell development are up-regulated.

healing of the mucosa. However, an earlier paper reports no difference in *TFF3* expression between normal and UC active mucosa [42].

In our investigation, we found that *TGFB1* was down-regulated in remission patients compared to normal. TGFB1 is negative regulator of mucosal inflammation, and it is well known that this cytokine is up-regulated in inflamed mucosa [43,44]. TGFB1 signals from the receptor to the nucleus through several proteins called SMAD's. Previous reports on the expression on TGFB1 in healthy and remission mucosa varies from no difference to down-regulated [43,45]. However, changes in *TGFB1* expression should be interpreted with caution due to extensive post-translational modifications necessary for activation of the TGFB1 protein.

Strength and weakness

The main strength of this paper is its focus on clinical remission in UC patients, highlighting the found perturbation as possible central factors in the basic immunopathology of Ulcerative colitis. There are weaknesses to the study as well: (A) The study population is clinically heterogeneous in that participants are in different phases of their disease and on a variety of medication, making it more difficult to discuss the mechanics of the pathways affected. However, this makes our results more clinically applicable to the average UC patient and not just the un-treated or the anti-TNF naïve etc.; (B) Only partial compliance with the MIQE guideline for PCR research as *ACTB* was sole reference gene. This can make the fold change results more uncertain, albeit most of our findings are in line with previous research and later validation showed *ACTB* to have low inter- and intragroup variation, thus, introducing little error. (C) Because of the invasive nature of the sample collection, our control population are people referred for colon cancer screening thus resulting age difference between study groups; (D) The low statistical power precludes models adjusting for medication, disease duration, and smoking status etc. (E) Transcriptional analysis has the inherent restrictions that it does not prove a functional protein, therefore any interpretation of difference on a protein level based on at transcriptional levels should be done with caution, nevertheless, it may serve as a hypothesis generator for further research. (F) We used two different methods of detection with qPCR. This is due to cost and time restrictions. Prefabricated plates saves time as we do not have to go through the time-consuming process of designing, optimizing and validating in total 29 new genes. In our exploratory context we believe this to be an acceptable approach.

Concluding remarks

In conclusion, we have shown that in clinical UC remission there is still an ongoing expression of inflammatory

mediators, although it seems to be more balanced towards mucosal healing. A mucosa with MES 1 transcribes more pro-inflammatory mediators than in MES 0, which may have clinical impact such as when to de-escalate treatment. Finally, we found that important transcription factors in the JAK/STAT pathway are still up-regulated in remission patients.

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Ethical approval

All participants were informed and signed a written consent. Approval including the use of biobank was granted by the Regional Committee of Medical Ethics of Northern Norway Ref no: 1349/2012.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Paper 2

PLOS ONE

RESEARCH ARTICLE

Real-life evaluation of histologic scores for Ulcerative Colitis in remission

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Abstract

Background

Histological evaluation of ulcerative colitis (UC) patients has been debated ever since the first description of the disease and its role in follow-up has never been fully established. Recent evidence suggests an added benefit in accuracy when evaluating if the patient is in remission. Unfortunately, there are several different histological indices, and it is difficult to compare outcomes where different scores are applied. Histopathological evaluation is prone to subjective biases, despite the use of indices. In addition, these indices are developed by expert IBD pathologist, but applied at large, by general pathologist. Therefore, we evaluated the three most applied histological indices for UC on samples from patients in remission to compare test qualities and estimate their usefulness to identify remission by both general and GI specialized pathologist.

Method

Mucosal biopsies from 41 UC patients in clinical and endoscopic remission were collected as part of a larger study on UC. Three pathologists blinded to the patients' clinical status evaluated them using Geboes score (GS), Nancy Index (NI) and Robarts Histopathological Index (RHI). We calculated the agreement between the pathologists using Inter-class correlation (ICC) and visualized it with ICC-plots and Bland-Altman plots. Association between clinical factors and histological category were analysed by Fisher's exact test.

Results

The ICC value for GS, RHI and NI were 0.85, 0.73 and 0.70 respectively. The limits of agreement were ±6.1, ±4.0 and ±1.4, for GS, RHI and NI, respectively. Mayo endoscopic subgrade and UC clinical score did not show association with any histological scores. Despite clinical and endoscopic remission 7–35% of the patients displayed histological inflammation on a level classified as active disease, depending on the index and cut-off.

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Conclusion

A substantial amount of UC patients in clinical and endoscopic remission display inflammation on a histological level, but the ability to classify these patients accurately and consistently could be improved.

Introduction

Ulcerative colitis (UC) is a chronic disease of the colon with relapsing-remitting characteristics. The introduction of targeted antibodies, such as anti-TNF, directed against key pro-inflammatory mediators, has improved patient outcome and lowered colectomy rates [1, 2]. However, the medication is expensive and has serious side effects like lowering the immune competency against certain infections and cancers [3]. Therefore, finding optimal criteria for remission is important, not only for the patients' health but also in a health-care economic aspect.

There is no universally applied definition of the state of remission, but usually only clinical or endoscopy-based scores are applied. The current treatment goal is partial Mayo score/SCCAI $\leq$1 and mucosal healing (MH) which is defined by Mayo endoscopic score (MES) of $\leq$1 [4–6]. However, this recommendation is moving towards MH to include only MES/Ulcerative Colitis Endoscopic Index of Severity (UCEIS) of 0 [7].

Histology adds a dimension in the evaluation of remission which can be beneficial. This was illustrated by a relapse prediction model that included both histologic and endoscopic activity. The model could predict relapse better than endoscopy alone [8]. Histology can detect subclinical inflammation despite endoscopically normal/near-normal mucosa and this inflammation increases the risk of an unfavourable outcome, such as relapse or neoplasia [9–13]. The European Crohn's and Colitis organization has recently published guidance on this topic [14]. However, the multiple scoring indices for histopathology in UC makes it difficult to compare the results between papers [15]. In addition, most of these indices lack thorough validation [16, 17]. Geboes Score (GS), Nancy Index (NI) and Robarts Histopathological Index (RHI) are the few that are partly/fully validated, and they vary in complexities and features they evaluate [18–20]. The position paper for ECCO recommends NI for clinical practice and observational studies. For histology to be of use in determining remission certain criteria must be fulfilled: A. It must add information of the inflammatory state not otherwise obtained. B. It must reliably and accurately identify these signs. C. The use yields a benefit in patient outcome. This paper focuses on the two first subjects, but also explores the relationship between histology grades and clinical parameters.

The US Food and Drug administration now recommends that histopathology should be included as endpoints in new trials. Therefore, there is an urgent need to define the histopathological remission state so it can be applied in trials and in the clinic. To address this, we evaluated the properties of the three most validated histological indices in a population defined to be in remission according to the current recommendations.

Material and methods

Study population

This study is a part of the Advanced Study of Inflammatory Bowel disease (ASIB) prospective study at the University Hospital of Northern Norway, Tromsø. All study participants gave

written, informed consent. The study and storage of biological material was approved of by the Regional Committees for Medical and Health Research Ethics, division North (REK Nord ID:2012/1349).

The selected participants were previously diagnosed with UC according to diagnostic recommendation [5]. Overview of baseline characteristics is presented in Table 1. Sample collection was performed at routine endoscopy for patients in remission from August 2013 to April 2016.The most frequent clinical indication being follow-up due to cancer screening and de-escalation of treatment. Inclusion criteria were age between 18 and 80 with clinical and endoscopic remission defined as Mayo clinical score/Ulcerative Colitis Clinical Score (UCCS) of 0 or 1 and Mayo endoscopic score (MES) of 0 or 1. Total Mayo score above 1 or rectal bleeding was not included. IBD medication was not an inclusion or exclusion factor.

Histology

All biopsies were formalin fixed immediately after sampling and embedded in paraffin. Multiple 3-μm sections were cut with a Micron microtome (HM355S, ThermoFisher, Tudor Rd, Runcorn WA7 1TA, United Kingdom) and stained with haematoxylin and eosin. In cases of multiple biopsies from one patient, the highest scoring biopsy was included in the analysis. Slides were investigated by three pathologists (SWS, SMD and LBR) blinded to the endoscopic score and biopsy location. SWS and SMD are general pathologists who evaluate 200–300 GI samples yearly, of which about 20–30 are IBD related. LBR works mainly with GI samples and sees around 180–360 IBS samples yearly. The final score for a biopsy is the average of the three pathologists. Two of the pathologists are located at the University Hospital of North-Norway while the third is located at Herlev Hospital in Denmark. SWS and LBR evaluated the slides using white light microscopy, while SMD evaluated the slides digitally, scanning them with Pannoramic 250 Flash III (3DHISTECH Ltd. Budapest, Öv u. 3, 1141, Hungary) at 40x with CaseViewer 2.3. In order to evaluate for intra-rater variability and explore the difference between light microscopy and digital microscopy SWS evaluated the slides a second time digitally with a 2-month interval. All pathologists were sent a scoring protocol to improve coherent rating.

The definition of remission across the three indices is not set, different studies have used different cut-offs. GS range from 13 to 7 (Table A of GS continuous vs original Table A in S1 Appendix) and RHI from <6 to ≤ 1 [21–26]. While the developers of NI suggest that ≤ 1 should be the cut-off, 0 is also applied in some papers [27]. As the cut-off values are debatable, it is of interest to explore the impact these definitions would have on a population in clinical/endoscopic remission. Therefore, we defined two separate definitions of histological remission,

Table 1. Baseline characteristics.

UC remission	
Number of patients	41
Gender(M/F)	16/25
Age(mean)	43
Biopsy location (Rectum/Sigmoid/Other)	22/13/6
Average endoscopic score (MES)	0.24
Average clinical score (UCCS)	0.15
Median Robarts Histopathological Index	1
Median Nancy Index	0
Median Geboes Score	4
Average Disease duration	8.8 years

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one strict and one relaxed. To be in line with previous research and to exclude mucosal neutrophils and basal plasmacytosis, the strict cut-off was GS <7, RHI <4 and no points allowed for neutrophils in neither epithelium nor lamina propria and NI = 0 [14, 27–30]. The relaxed cut-offs for remission for NI and RHI are the developer's recommendation (NI <2, RHI <6). For GS, the relaxed cut-off is widely applied (GS <13) [30].

Statistics

All statistics were performed with Rstudio Version 1.2.5019. Inter/intra-rater calculation was done with the "irr" and "KappaGUI" packages. Inter-rater on ordinal/continuous variables was performed with two-way random, average score, intraclass correlation coefficient (ICC) for consistency (C,3). Intra-rater was performed with two-way random, single score ICC for absolute agreement (A,1). On categorical variables Fleiss' kappa was applied and evaluated according to Landis et. al: < 0: Poor agreement, 0.01–0.20: Slight agreement, 0.21–0.40: Fair agreement, 0.41–0.60: Moderate agreement, 0.61–0.80: Substantial agreement, 0.81–1.00: Almost perfect agreement [31]. Bland Altman plots were calculated with mean squared error according to method proposed by Mark Jones et.al [32]. All scores were standardized by dividing them on their theoretical max and then transformed with square root because of skewness in the raw data. The standardization makes limits of agreement (LOA) directly comparable. We investigated systematic rating differences between raters with Kruskal-Wallis rank sum test. If significant, we made a sub-analysis to identify which graders were different. The sub-analysis was performed as pairwise comparisons using Wilcoxon rank sum test with multiple comparison adjusted p-values (Benjamini and Hochberg). Relationships between two dichotomous variables was assessed with chi-square test or Fisher exact test, dependent on group sizes. These statistical tests were performed with "rstatix" package for R.

Results

In total 41 biopsies from 41 UC patients in clinical and endoscopic remission were evaluated by two general pathologists and one GI-specialized pathologists using all three scoring indices. Only five biopsies were evaluated as 0 by all three pathologists across all three indices. Median scores for indices were 7, 4 and 1 for GS, RHI and NI, respectively. Between 7 and 15% of all the samples still exhibited histological activity to such a degree that they would be classified as active disease with a relaxed histological remission definition (GS<13, R<5, N<2). With a stricter remission definition, the share of active disease increases to between 22–32% of all samples (GS<7, R<4, N<1). There was a systematic difference between the three pathologists, where LBR rated higher on average than SWS and SMD with GS, but with a similar standard deviation (S1 Table). This was significant in a Kruskal-Wallis rank sum test for both GS and RHI (S1 Fig and S2 Table)

Agreement between raters

The inter-rater ICC value for the features vary from poor(<0.50) to excellent (>0.90) according to the classification suggested by Koo et al. (Table 2) [33]. Features describing severe inflammations are over/under-estimated due to small sample size for those features. Only GS achieves an agreement of good, while RHI and NI achieves moderate agreement. The intra-rater evaluation displayed better results, as the final score for the three indices ranged from good to excellent (0.78–0.92, Table 2). Fig 1 is an ICC plot illustrating inter-rater agreement between raters for each slide on the Final score for each index. Modified Bland-Altman (BA) plots displayed the limit of agreement as ±0.53, ±0.59 and ±0.35 for GS, NI and RHI, respectively (Fig 2). If transformed back to the original values it corresponds to ±6.1, ±1.4 and ±4.0.

Table 2. ICC values for histological feature agreement.

Geboes Score	ICC Inter	ICC Intra	N. patient*
Grade 0 Structural architectural changes	0.65 (0.42–0.80)	0.95 (0.91–0.97)	26
Grade 1 Chronic inflammatory infiltrate	0.83(0.71–0.90)	0.78(0.62–0.88)	26
Grade 2A Eosinophils	0.65 (0.42–0.80)	-0.04(-0.33–0.26)	15
Grade 2B Lamina propria neutrophils	0.77(0.61–0.87)	0.89(0.80–0.94)	4
Grade 3 Neutrophils in epithelium	0.89(0.81–0.94)	1.00	4
Grade 4 Cryptdestruction	-0.03(-0.73–0.42)	0.00 (-0.30–0.30)	3
Grade 5 Erosion/ulcus	0.10 (0.51–0,49)	NA-	3
Final Grade	0.85(0.75–0,91)	0.96 (0.93–0.98)	35
Robarts Histopathological Index			
Chronic Inflammatory Infiltrate	0.83 (0.71–0.90)	0.77(0.62–0.87)	26
Lamina propria neutrophils	0.77(0.61–0.87)	0.79(0.64–0.88)	4
Neutrophils in epithelium	0.89(0.81–0.94)	1.00	4
Erosion/Ulceration	0.09(0.53–0.48)	NA-	3
Final Grade	0.73(0.54–0.85)	0.96(0.93–0.98)	26
Nancy Index			
Chronic inflammatory cell	0.42(0.02–0.67)	0.38 (0.10–0.61)	5
Acute inflammatory cells	0.79 (0.64–0.88)	0.86 (0.75–0.92)	10
Ulceration	-0.04(-0.74–0.41)	NA	2
Final Grade	0.70(0.50–0.83)	0.86(0.75–0.92)	13

*Number of patients with a score >0.

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There is a tendency of higher agreement in the extremes of the scores, albeit not a big difference.

Remission aid and clinical application

Next, we evaluated the inter-rater properties with two different cut-offs for remission, relaxed (GS <13, RHI <5, NI <2) and strict (GS <7, RHI<3 and NI <1). The latter definition resulted in a doubling of patients defined with active disease with GS and NI but no change in RHI (S2 Fig). Both cut-offs showed similar kappa values, from fair to moderate agreement (Table 3). NI and RHI performed slightly better than GS with strict cut-off.

Thereafter, we investigated if there was a difference between high (MES = 1 and UCCS = 1) and low (MES = 0 and UCCS = 0) endoscopic and clinical grade and the histological category (Active or Remission). Neither clinical grade, nor endoscopic grade showed significant dependence with the histological category, regardless of strict or relaxed cut-off (S3 Table).

To control for potential confounding factors, we investigated difference in histology score by their biopsy location and IBD medication. The distribution was rectum (n = 22), sigmoid (n = 13), and other (n = 6). The Kruskal-Wallis test showed no significant difference between the different locations (S3 Fig) and the Wilcoxon rank sum test showed no effect of different medication on the histological scores (S4 Table).

Discussion

This observation study evaluates the performance of the three most validated histological scores for UC in a remission setting. The main findings are a poor to excellent inter-rater agreement between the three histological scores, as well as a fair to moderate inter-rater agreement for determining remission. The patients were defined as remission patients according to

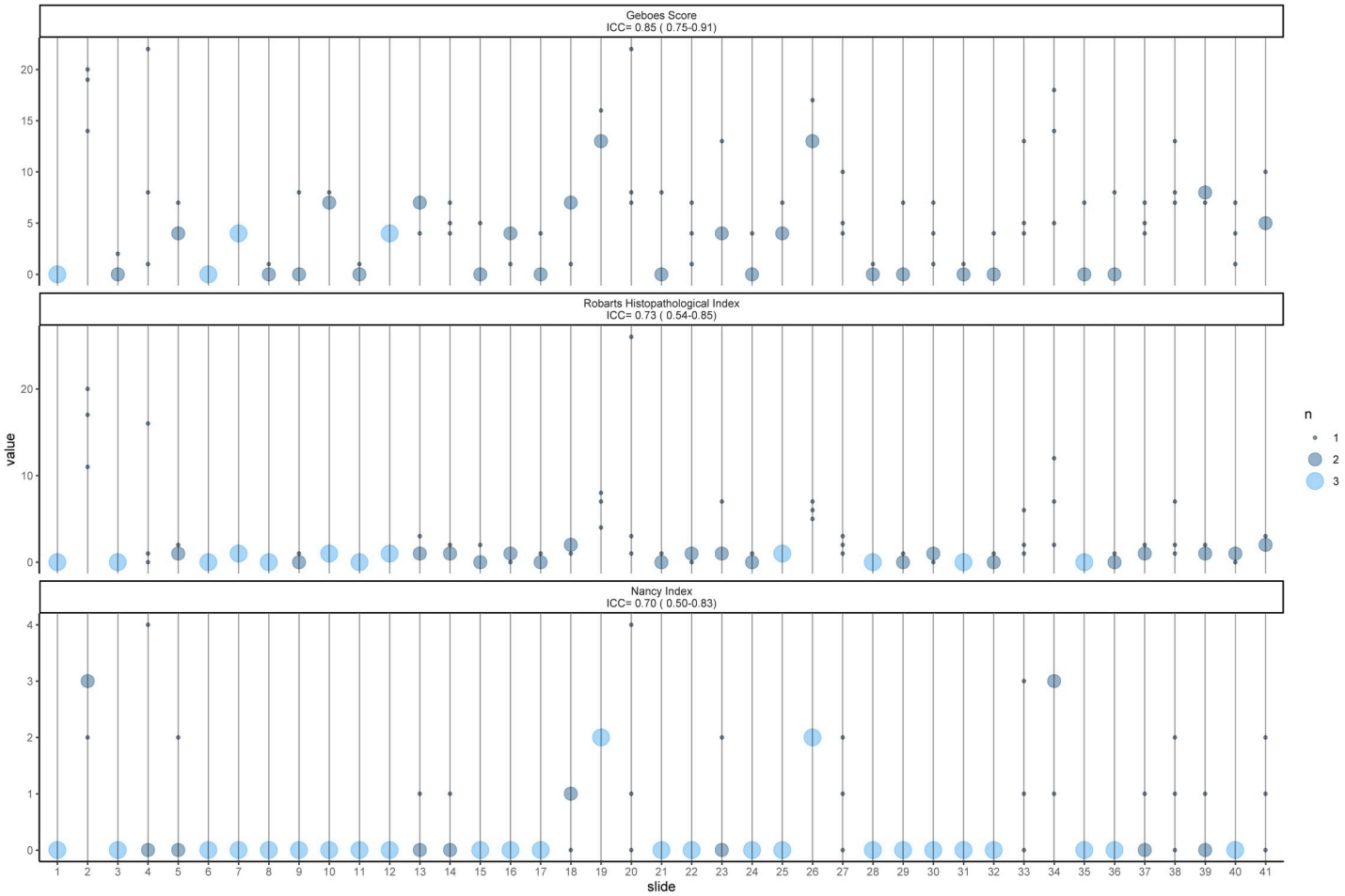

Fig 1. ICC dot plot. The plot illustrates how the Final grade for each slide is scored by the three pathologists. The size of circle indicates how many raters gave the same score.

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the current guidelines (i.e. clinical and endoscopic remission). Nevertheless, a substantial number showed histologic inflammatory activity, indicating that histology can unveil inflammatory features in a population of patients in remission pre-selected on clinical and endoscopic findings. This is in line with previous publications [34, 35].

Compared to previous research, our results show lower concordance between raters. Jairath et al. had inter-rater ICC of 0.88, 0,86, 0,80 for GS, RHI and NI respectively [36] and Marchal-Bressnot et al. achieved a ICC value of 0.86 when developing the NI [20]. Mosli et al. achieved 0.82 when developing RHI [19]. The GS method paper applied pairwise Cohen's kappa and is not comparable with our results [18]. This difference could be either the result of different interpretations of scores between our raters or observational errors. All raters were sent the same scoring protocol (S1 Appendix) to improve coherent rating. It could be argued that a scoring protocol is not sufficient to ensure coherent rating from general pathologists. We argue that this the actual situation in most hospitals outside specialized tertiary centres. Thus, our results show the real-life utility of the scores. By including nothing but patients in remission, only the lower range of the histologic scales are represented, and this may be viewed as a "stress test" of the scores for this specific patient group. Consequently, lower inter-rater agreement is expected.

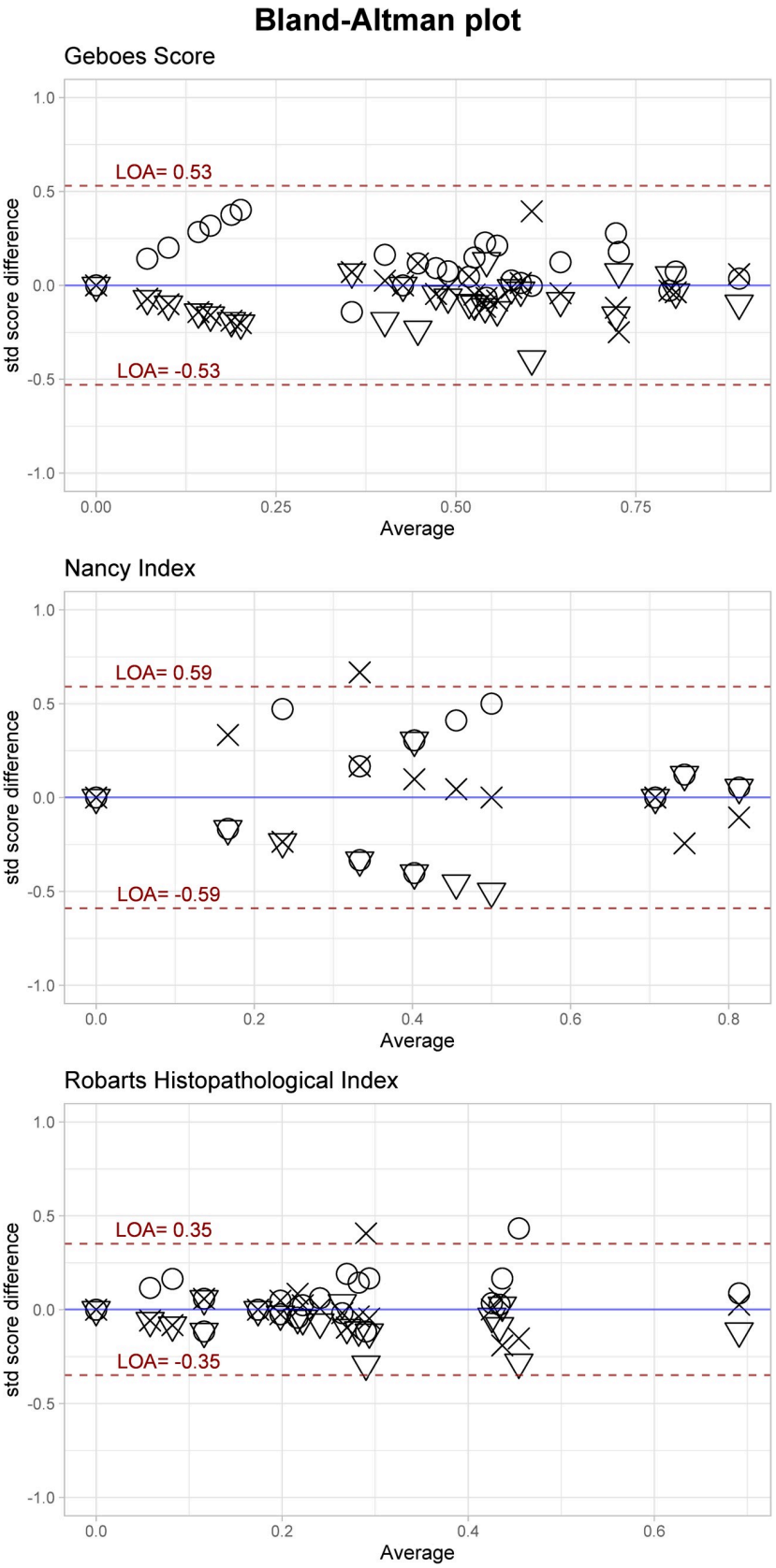
Bland-Altman plot
Geboes Score
LOA= 0.53
LOA= -0.53
std score difference
Average
Nancy Index
LOA= 0.59
LOA= -0.59
std score difference
Average
Robarts Histopathological Index
LOA= 0.35
LOA= -0.35
std score difference
Average

Fig 2. Modified Bland-Altman plot. The plot indicating the limits of agreements for the Final grade for each index. The plot shows less dispersion in the high and low average values, indicating higher agreement. The absolute scores were standardized then transformed by the square-root. This makes the LOA directly comparable.

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The results show discrepancies in the severe inflammatory features due to low number of samples. This is not the case for the eosinophilic feature of GS and the structural architectural changes features. The number of eosinophils vary greatly between subjects depending on age and location in a healthy colon [37]. There is no recommendation for what an acceptable cut-off for eosinophils per segment of colon in UC remission is, and therefore, evaluating this feature coherently is challenging. Normal variation is also one of the challenges when evaluating structural architectural changes. Due to the inherent number of histological features included in this grade such as crypt branching, mucin depletion etc., an overview of it will leave too many features to subjective interpretations. Especially, since "grade 0.0 is indicated the absence of any abnormality.", which is almost never the case. It could be interpreted as disease specific abnormality, but the need for subjective interpretations on numerous features will challenge coherency between raters. GS is the only index to include such a feature.

Another subgrade that scored low in the inter-rater score is the Nancy Grade 1, chronic inflammatory cells. It is difficult to distinguish between moderate to severe amount of chronic inflammation from acute inflammatory features. There are seldom signs of severe chronic inflammation without concomitant presence of neutrophils, which defines the criteria of grade 1: "Grade 1 corresponds to the lack of mucosal neutrophils, a pivotal marker of disease activity, even though moderate or severe chronic inflammation can be present" [20]. Thus, making it a cause for variation and in many cases redundant.

Our intra-rater evaluation was as good or better than the inter-rater values, except for the eosinophile feature. Interestingly, there was a clearer difference between the raters than between modalities (white light microscopy or digitally scanned slides), suggested by the intra-rater results and the Kruskal-Wallis test (S1 Fig). This indicates that these methods can be used interchangeably for NI and RHI which does not evaluate eosinophils specifically. The high intra-rater score and the relatively low inter-rater score indicate that a central raters approach to IBD-pathology could be beneficial. Standardization of extraction, preparation and scanning is easier to achieve than extensive training of pathologists.

The modified Bland-Altman analysis identified the same as the Kruskal-Wallis analysis, that one pathologist rates higher than the two others, nevertheless the standard deviations are similar (S1 Table). The obvious explanation the IBD-related experience difference between LBR and the general pathologists. This indicating that experience gives a different understanding of the scores, which seems to effect accuracy but not necessarily precision as the IQR/SD are similar between raters. The LOA gives the amount the raters can be discordant with the mean estimated score. The results show better agreement for RHI than NI and GS. This could be a result of RHI being developed from GS by selecting the features of best agreement. If we evaluate the absolute LOA scores it shows that all the scores are rather insensitive to minor

Table 3. Inter-rater agreement evaluated with Fleiss' kappa.

	Strict	Relaxed
GS	0.30	0.57
NI	0.44	0.44
RHI	0.48	0.47

GS, Geboes Score; NI, Nancy Index; RHI, Robarts Histopathological Index.

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differences, this can explain the drop in agreement when dichotomizing the indices from continuous variables to "Active" and "Remission". The agreement appears to be better in the low and high average scores for all three indices. An explanation could be that it is easier to rate the extremes of the distribution rather than the middle. This is unfortunate as the cut-off for remission is in the low-middle of the distribution.

In our data we defined two cut-off values for histologic remission in order to investigate whether there would be a difference between the two groups in relation to other clinical features of importance. There was no dependence between the clinical scores (MES and UCCS) and histologic category for any of the indices. This is important because a high degree of dependence between clinical scores and histology would render one of the factors redundant, as one factor could predict the other. By being independent they can complement each other Previous studies are conflicting in their report of this relationship between clinical scores and histologic scores [9, 12, 18, 21, 38].

Strengths and limitations

Our study has several limitations, first and foremost is the different modalities used to evaluate the slides and the blinding to the biopsy location. The evaluation of eosinophils was challenged by two factors, one was the blinding of biopsy location to the pathologists and the different modalities of observation for the intra-rater analysis. Eosinophils significance in UC can be debated as the two recent indices does not it include it in a separate category and marked increase in eosinophils without other inflammatory cells suggest eosinophilic colitis and not UC. Despite the poor intra-rater value for eosinophils, the other categories had good intra-rater ICC, which suggests that error introduced by scanning the samples is small. Unfortunately, our biopsies are not orientated after collection so the pathologists could not reliably evaluate basal plasmacytosis, defined as plasma cells between the base of the crypts and *muscularis mucosae* [39]. Nevertheless, plasma cells fall under the category of chronic cell infiltrate, which is evaluated in all indices. One could argue that a scoring protocol is insufficient education to achieve accurate evaluation by a general pathologist.

Our strengths are the approximation of real-world setting where patients are under different treatment regimens and in different clinical settings, making any finding representative for the IBD remission population.

Conclusion

Our study evaluated reliability of histology scores, in order to estimate their usefulness in clinical decisions. We found that there is a moderate to good agreement between raters when using three of the most common histological scoring indices, but with a LOA that could be improved. Unfortunately, when dichotomising the scores into active and remission the agreement falls to fair and moderate. Therefore, without more extensive training or the adoption of a central raters approach using the current histological indices for deciding remission should be done with caution.

Supporting information

S1 Fig. Difference between raters. Significant difference between raters were tested with Wilcoxon rank sum test with Benjamini-Hochberg adjusted p-values.
(PDF)

S2 Fig. Bar plot. Difference in patients classified as remission or active according to relaxed or strict definition of remission.
(PDF)

S3 Fig. Histologic score by biopsy location. No difference was found between biopsy locations.
(PDF)

S1 Table. Descriptics for raters.
(DOCX)

S2 Table. Kruskal-Wallis rank sum test on raters, by indices.
(DOCX)

S3 Table. Fisher exact test between the UCCS and MES scores and histologic category. The table shows that histological category is independent for whether the sample was collected from a MES/UCCS 0 or 1 patient. This was true for both Strict and Relaxed category.
(DOCX)

S4 Table. Wilcoxon rank sum test on the effect of medication on histological scores.
(DOCX)

S1 Appendix. Scoring aid provided to the pathologists.
(DOCX)

S1 Data.
(CSV)

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Paper 3

IFNG:IL33 ratio predicts relapse in UC remission patients.

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Abstract

Background: Despite the continuous search for better treatment and surveillance regimes for Ulcerative Colitis (UC), a substantial number of patient experiences relapse. The current management usually includes only three modalities: symptoms, endoscopy and faecal calprotectin. To detect imminent relapse and improve treatment other modalities could be included such as histology and transcriptional biomarkers. This study investigates the predictive abilities of factors related to symptoms, endoscopic appearance, histological appearance, and transcriptional assays.

Method: 41 participants in clinical and endoscopic remission of UC were included in this retrospective cohort. Mucosal biopsies were collected for histological and transcriptional evaluation. Histology was evaluated by two general pathologists, and a third GI-specialized pathologist refereed on disagreement. Targeted gene transcription analyses were performed using hydrolysis probes.

Results: Of the 41 participants, 14 experienced relapses during the follow-up time with a median time to relapse of 6.5 months (IQR 6.6) .Median follow-up time was 8 months for the study Eight of 42 investigated variables showed non-zero coefficients in a LASSO regression analysis. Of these eight, the best performing factor was a ratio between IFNG and IL33 gene expressions. A univariate cox regression analysis showed 5.3 times (95%Cl = 1.8-15.4) higher risk of relapse in a low IFNG:IL33 ratio group than in a high group. The IFNG:IL33 ratio performed better than both UCCS and MES in predicting relapse. Histologic evaluation could not predict relapse.

Conclusion: A ratio between IFNG and IL33 gene expression is predictive of relapse and could be a potential new biomarker for relapse in UC remission patients.

Introduction

Ulcerative Colitis (UC) is a chronic inflammatory disease of the colon, and it is typically characterized by a relapsing-remitting course. Previously, the main costs were linked to hospitalization, but the last two decades it has shifted towards medication due to arrival of expensive biological agents (1). Although, high in costs, biological agents have proved beneficial in lowering colectomy rates and maintaining remission (2, 3). Recent studies report that 43% have a relapsing and remitting course during the first 12 months following diagnosis, and 16% relapse within 1 year of de-escalation of biological treatment. In a 10-year perspective 67-83% relapse dependent on the clinical situation (4, 5). Therefore, it is of interest to identify UC patients at risk of relapse so that treatment can be optimized to ensure longer periods in remission and fewer relapses using minimal but adequate therapy.

There is little knowledge about what initiates a relapse. Most research is focused on targets for treatment and to a lesser degree on surveillance markers. The problem with this approach is that it does not differentiate between the mechanisms of resolution and initiation of inflammation. Factors that can indicate when inflammation is resolved are not necessarily the same as the ones that can give an early warning of an impending relapse.

An initiative by experts under the auspice of International Organization for the Study of Inflammatory Bowel Diseases (IOIBD) recommends a set of goals across different modalities in order to achieve optimal treatment (6, 7). According to this expert panel only symptoms, endoscopy and faecal calprotectin are recommended for surveillance of UC. These three markers can detect manifest inflammation, but so far, it is not found that they can detect imminent relapse.

The aim of the present study was to address this by an exploratory survival analysis on UC patients in remission, with factors across four different modalities (symptoms, endoscopy, histology and transcripts) in search of potential new biomarkers for relapse.

Material and methods

This study was a part of the Advanced Study of Inflammatory Bowel (ASIB) prospective study at the University Hospital of Northern Norway, Tromsø. All study participants gave a written, informed consent. The study, including storage of biological material, was approved of by the Regional Committees for Medical and Health Research Ethics, division North (REK Nord ID:2012/1349).

Study Participants

41 UC patients in remission were recruited from the Advanced Study of Inflammatory Bowel Disease (ASIB). UC was diagnosed according to established diagnostic definitions (8). Patients in clinical remission were recruited at three different time points: at a routine follow-up, at a 6-month control after initiation of biological treatment or at an evaluation for terminating biological treatment. Independent of medication only patients with confirmed remission defined as: UCCS/Mayo clinical score of 0 or 1, with mayo endoscopic subscore of 0 or 1, no points were allowed on rectal bleeding feature and a total Mayo score larger than 1 was not included (8). The current treatment regime was recorded at time of inclusion. The different steroid and anti-TNF treatments where grouped together into categorical yes/no variables. Relapse was defined as contact with health-care provider and escalation of treatment, regardless if the escalation was an increase in dosage of mesalazin or addition of biological treatment.

Histophatology

All biopsies were immediately fixed in 10% formalin after sampling and embedded in paraffin. Multiple 3-µm sections were cut with a Micron microtome (HM355S, ThermoFisher, Tudor Rd, Runcorn WA7 1TA, United Kingdom) and stained with haematoxylin and eosin. Slides were investigated by two general pathologists blinded to the endoscopic score and biopsy location. If there were disagreements a third gastrointestinal (GI)-specialized pathologist gave the final score. Each slide was evaluated with the three most applied histologic UC indices, Geboes Score (GS), Robarts Histopathological Index (RHI) and Nancy Index (NI) (9-11). The histological evaluation method is further described in a previous publication (12). Histologic strict definition of remission was GS <2A.1, RHI <4 and NI = 0 and histologic relaxed definition was GS <3.1, RHI<5 and NI<2

Quantitative Polymerase Chain Reaction (qPCR)

Quantification of genes expression was performed as close to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines as possible (13). The geometric mean between *HPRT1* and *RPLP0* was used as reference gene for the SYBR-Green assays, and *ActB* was used for the hydrolysis probe assays. More detailed description of qPCR method can be found in a previously published paper (14). The teste genes were *IL1B, IL6, IL8, IL10, IL18, IL21, IL23, Il33, TNF, TGFb, IFNG, TLR4, ST2, SPI1, TBX21, FOXP3, GATA3, RORC, ACTB, IL17, IL4*. Corresponding primer and probes are listed in supplement 1

Immunostaining

Endoscopic biopsies from the sigmoid colon of 9 UC patients in remission and 10 normal controls were analysed.

Formalin-fixed, paraffin-embedded 4 µm sections were deparaffinised and rehydrated through graded steps of xylene and alcohol. Antigen retrieval solution (DAKO, Glostrup, Denmark) was used and the sections were boiled in a water bath for 20 minutes, followed by 20 minutes cooling at room temperature. Goat serum 10% was used for blocking (20 minutes) prior to primary antibody incubation. Monoclonal antibodies for IL33 [1ug/ml] (anti-mouse, Nessy-1, Enzo Life Sciences) and vWBF[1/100] (anti-rabbit, Abcam) were incubated overnight at 4 C. Secondary goat antibodies conjugated with alexa555 or alexa647 for rabbit or mouse (Life technologies) were used as appropriated at [1/1000] and incubated for 90 minutes at room temperature. Hoechst 33258 (Life technologies) was used for nuclear staining. Sections were mounted with Fluoromount aqueous mounting medium (Sigma Aldrich/Merck,St.Louis, USA). Isotype and concentration matched antibodies were used (IgG mouse, rabbit, Cell signal Technology, Danvars, MA). Tonsillar tissue served as positive controls.

A Zeiss LSM780 CLSM microscope (Carl Zeiss Microscopy, Zena, Germany) was used with the Zen 2012 software (black edition) for taking images. Three representative images at x20 magnification were taken of each section. Nuclear IL33 signal was analysed using the Volocity® 6.3 software (Quorum Technologies Inc., Puslinch, Ontario N0B 2J0) using positive fluorescent signal of total nuclear area/positive nuclear area. Image processing was performed with Adobe Photoshop CC (Adobe System Software, Ireland Ltd, Dublin) with histogram adjustments only applied for whole images.

Statistics

Missing data were imputed using nearest neighbour averaging method. Variables with more than 20% missing values were excluded for analysis.

The data set had more variables than cases which challenge the ordinary approach of backward/forward selection for identifying potential predictors, as they run the risk of producing an overfitted model (all variables are listed in Table 4 in Supplements 1). To avoid this we applied a strategy often used for high dimensional data such as micro-arrays and sequencing data (15, 16).

To narrow down the number of potential covariates for the model building, a LASSO regression was run 10 000 times with different seed each time. This resulted in a range of different penalizing factors, which again produces a range of covariates with non-zero coefficients, i.e covariates with potential significance. In this exploratory study the lowest penalty i.e the lowest lambda (λ) value of the range, was chosen to avoid excluding potential covariates. The LASSO regression was done with glmnet package for R

The non-zero covariant then went through a forward stepwise model selection by AIC and univariate cox regression to identify covariates for a relapse-predicting model. In order to not violate the "10 events pr covariate" rule of thumb two of the most significant variables were transformed to a ratio. The optimal cut-off for relapse prediction was determined with a ROC analysis and Youden J statistics. This cut-off what used to dichotomize the ratio into a high and a low category, which then was analysed with Kaplan-Meier method to evaluate and illustrate difference in relapse-free survival between the two categories.

The calculations were done with Rstudio Version 1.3.1056. Packages used are listed in supplement 2

Results

Of the 41 patients in UC remission did 14 experience a relapse during the follow-up period. The median remission time was of 6.5 months (IQR 6.6), whilst those who did not experience relapse had a median follow-up period of 12 months (IQR 11). A total of 42 variables across clinical, medication,

histopathological and gene expression categories were evaluated. Among the clinical characteristics at baseline there were no statistical differences between those who relapsed and those who did not on p<0.05 level (table 1)

Table 1 Baseline data. Ulcerative Colitis Clinical Score (UCCS), Mayo Endoscopic Score (MES), Geboes Score(GS), Nancy Index(NI), Robarts Histopathological Index(RHI)

Variable	Relapse n=14	Remission n=27	p
Age (y), mean±sd	44.9±12.5	38.7±13.3	0,173
Sex: female	9(64%)	16(59%)	0,77
Disease duration (mth)	103±101	96±71	0,978
Biopsy location	7(50%)/4(28%)/3(22%)	15(55%)/9(33%)/3(11%)	0,593
UCCS	0.28±0.46	0.11±0.32	0,171
MES	0.42±0.51	0.14±0.36	0,0524
GS	5.64±4.4	4.62±4.36	0,456
NI	0.5±0.75	0.44±0.8	0,725
RHI	2.0±2.3	1.9±3.6	0,447
Current medication			
Mesalazin	13(93%)	25(92%)	1
Steroids	1(7%)	0	0,181
Methotrexate	1(7%)	5(18%)	0,346
Anti-TNF agents	10(71%)	17(63%)	0,604
AzathioprineMCP	6(42%)	11(40%)	0,91

Variable selection

After 10 000 iteration 8 variables had been identifies as non-zero by LASSO regression (table 2).

Table 2 Non-zero covariates. Result for 10 000 LASSO iteration. 8 covariates with number of times the algorithm resulted in a non-zero coefficient and the mean coefficients.

Variable	Number of Non-zero	Mean coefficient	sd	IQR	se	lower.ci	upper.ci
IL33	2987	0,179	0,131	0,229	0,002	0,174	0,184
Geboes Remission Relaxed	1204	0,506	0,236	0,320	0,007	0,493	0,519
IFNG	1135	-0,125	0,042	0,045	0,001	-0,127	-0,122
UCCS	1115	0,177	0,041	0,023	0,001	0,175	0,180
IL1B	1085	0,071	0,031	0,044	0,001	0,069	0,073
MES	1053	0,186	0,084	0,120	0,003	0,181	0,191
Age	743	0,005	0,003	0,004	0,000	0,004	0,005

Disease duration	3	0,000	0,000	0,000	0,000	0,000	0,000

Disease duration and age were excluded because of small coefficients. The six remaining variables were evaluated in univariate forest plot (figure 1) and with forward selection to uncover which of the covariates that were best suited for a final relapse model. In the univariate cox regression Geboes Remission Relaxed displayed an infinite coefficient due to no events in the active category and was therefore removed. *IL33*, *IFNG* and MES were the variables with the lowest AIC in a forward stepwise regression (Table 3 in supplement 1). The continuous variables were calculated into quartiles. Because of the opposite effect of *IFNG* and *IL33* we subtracted the *IL33* dCT value from the *IFNG* dCT, corresponding to a ratio (IFNG:IL33) in linear values. The ROC analysis of this ratio resulted in an optimal cut off of 4.2, which had a specificity of 92.6% and a sensitivity of 57.1% with an AUC of 76.5. With this cut-off the PPV and NPV were 80% and 80.6%

Figure 1 Forest plot. Displaying the five covariates from variables selected by LASSO regression. Reference levels not displayed. MES:Mayo endoscopic score, UCCS: Ulcerative Colits Clinical Score. All mRNA values are dCT values against reference gene value.

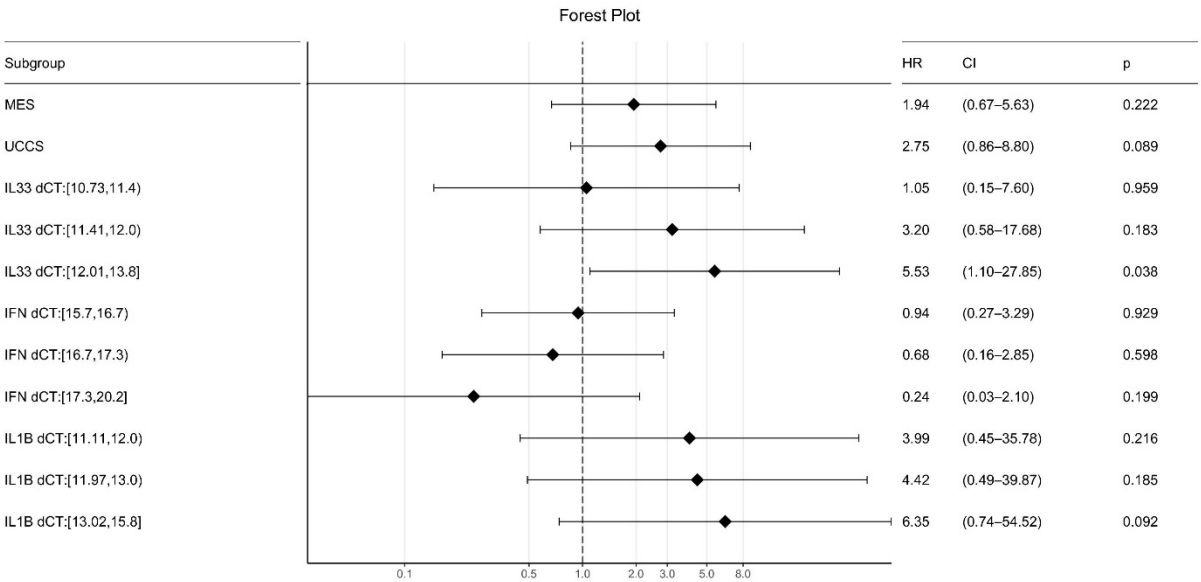
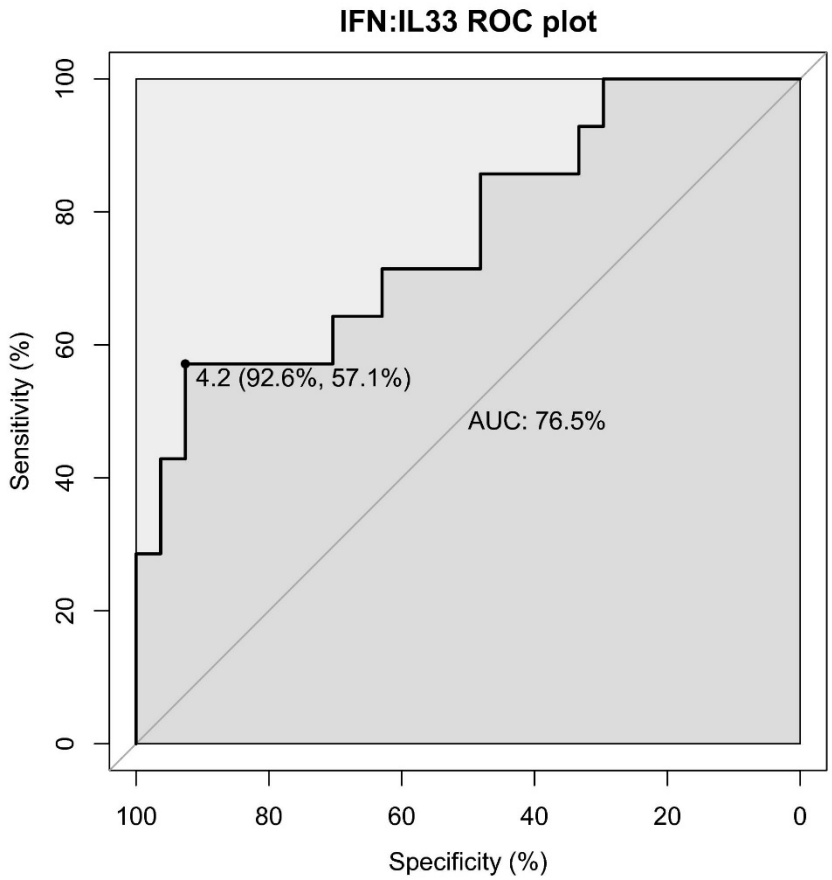


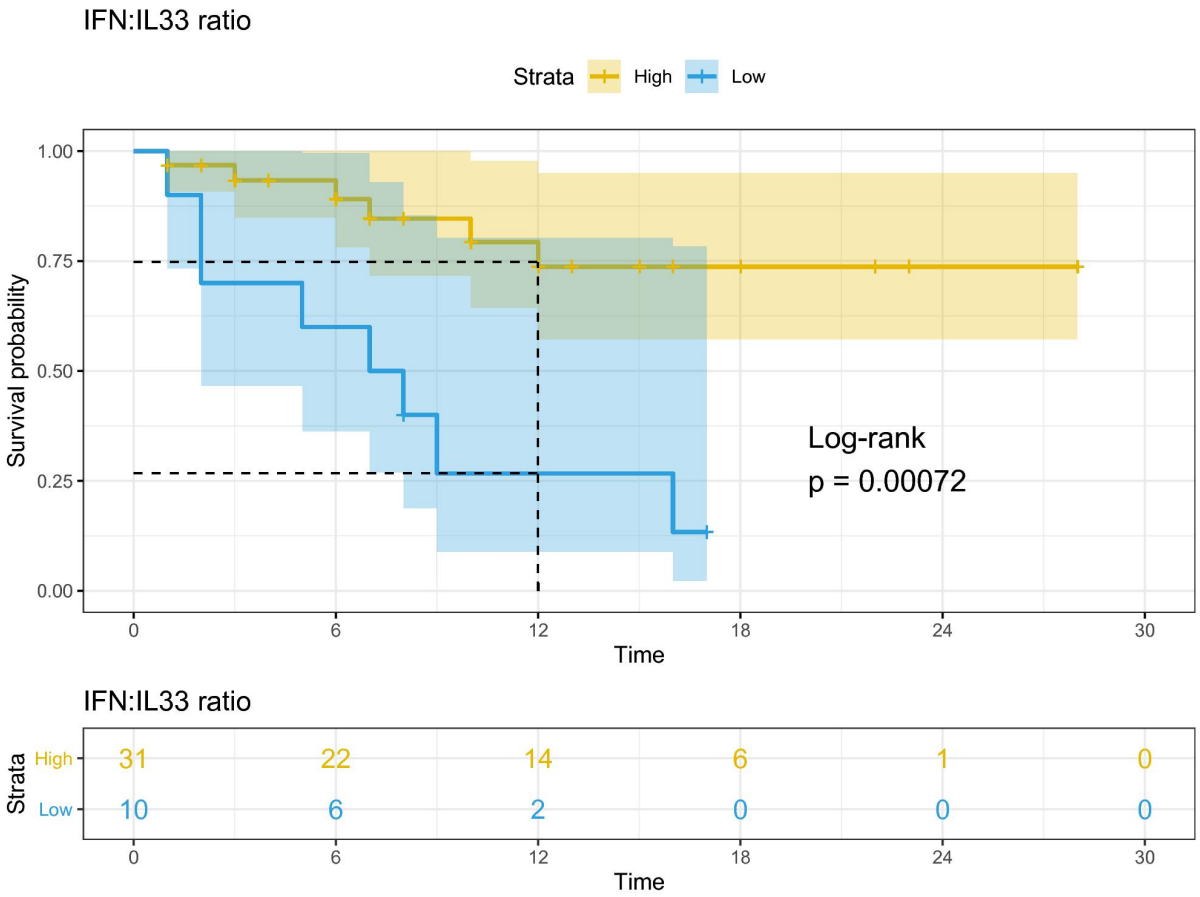
Figure 2 ROC plot of IFNG:IL33 ratio. The plot displays the optimal cut-off for maximal sensitivity and specificity calculated with youden statistics



Survival analysis

The Kaplan-Meier analysis on the dichotomized the IFNG:IL33 ratio (low ratio <= 4.2 and high ratio >4.2) revealed a statistical significant better outcome for participants with high ratio compared to the low ratio group (log rank test, p-value of $p < 0.001$). The one-year cumulative relapse was 26.7% in the low ratio group and 74.8% in the high ratio group.

Figure 3 Survival plot of the IFNG:IL33 ratio. Kaplan-Meier plot illustrating the difference in survival probability between a high and low ratio of IFNG:IL33 and tested with a log-rank test. Dotted lines represent 12 months survival.



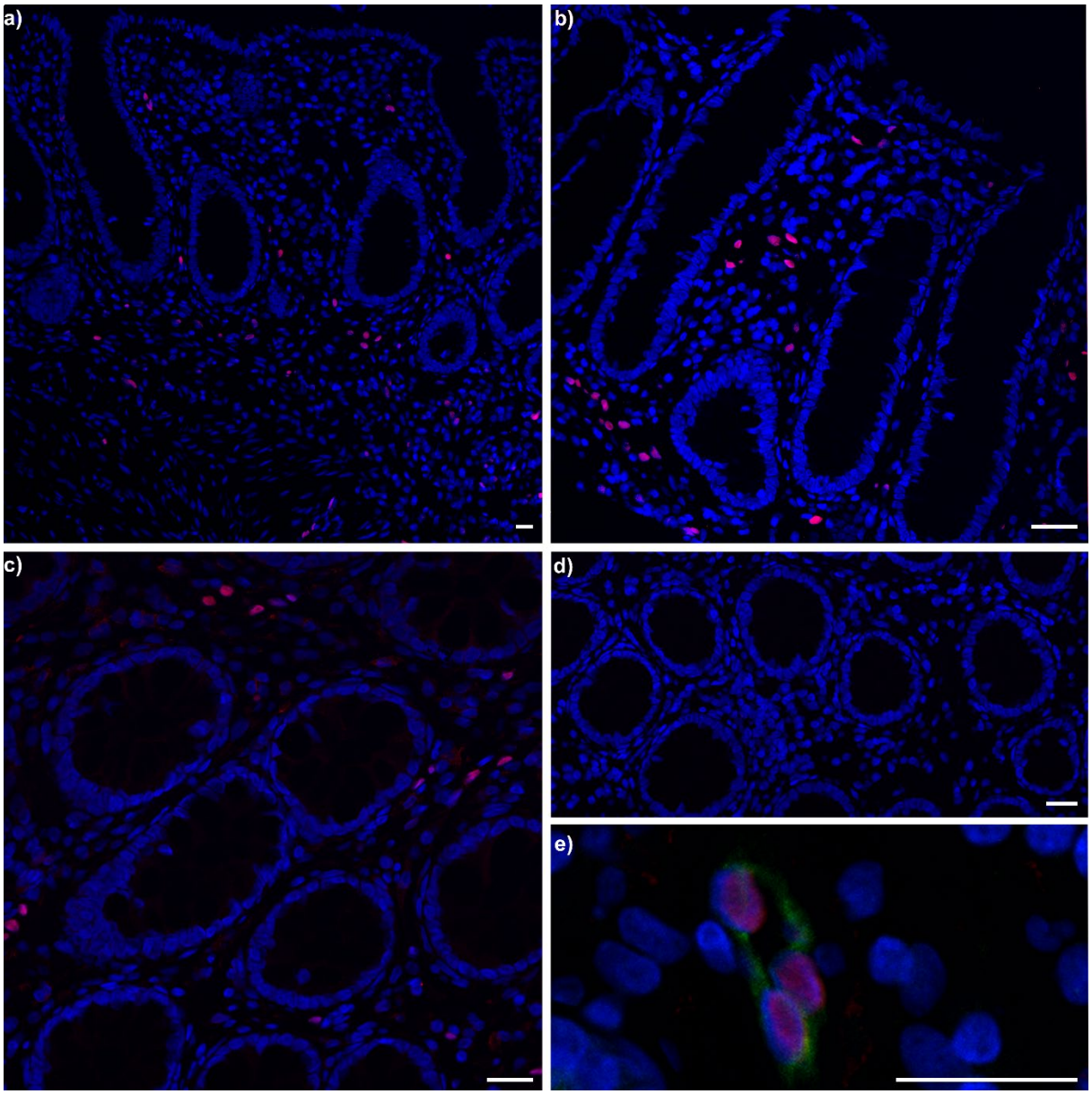
The IFNG:IL33 ratio was a better predictor than both endoscopic score and clinical score (figure 3 in supplement. The univariate cox analysis showed 5.3 times (95%CI = 1.8-15.4) higher risk of relapse in the low group than in the high group. In a multivariate model that includes sex, age and biopsy location the model, IFNG:IL33 ratio was still significant (HR = 4.9, 95%CI = 1.6 -15) although, the likelihood ratio test was not significant ($X^2(5)$ =10.75,p=0.06). A ratio of IFNG:IL1B were also significant but lower with a lower HR than the IFNG:IL33 ratio (HR = 4.5, 95%CI = 1.3-16.3).

As *IL33* showed an association to relapse its relationship with its receptor *IL1RL1* was investigated. *IL33* and *ILRL1* showed positive correlation, but there was no difference between those who relapsed and those who did not, figure 4 in supplement 1.

Immunostaining confirmed the presence of nuclear IL33 in both UC and normal controls. IL33 was present in endothelial cells (as shown in figure 4 costained with vWBF) and

mononuclear cells in the lamina propria. No intestinal epithelial cells were positive for IL33, neither in UC nor in the control group. Statistical analysis did not reveal a significant difference between the two groups. Furthermore, no clear difference in IL33 pattern was observed between relapse and non-relapse.

Figure 4 Immunostaining of Ulcerative colitis in remission. Formalin-fixed paraffin embedded colonic biospies from patients in UC remission (a-e). Immunofluorescence showing IL-33 (red) and nuclei stained with Hoechst (blue). IL-33 is located in the nuclei of cells located in the lamina propria(a,b,c), including endothelial vessels as shown in e) with endothelial vessels stained with von willebrandsfactor (green). No positive IL-33 cells were seen in the epithelium. Scale bars equal 25µm. A negative isotype and concentration matched control is shown in d).



Discussion

In this study we found a 5.3 times increased risk of relapse in UC patients with a low ratio between *IFNG* and *IL33*. There was no apparent difference in IL33 distribution in the mucosa between relapsers and non-relapsers. Histology could not predict relapse in our data.

The IFNG:IL33 ratio has not been previously described as a predictor of relapse in UC. The beneficial ratio consists of relatively lower gene expression for *IFNG* compared to *IL33*. IFNG is typically

described as a central cytokine in active CD (17-19), therefore it was surprising to find it as a risk factor for relapse in UC remission. Although, some studies have found it to have a role in both types of IBD (20-22). In general IFNG is considered to be central mediator in innate immune response, and in IBD it is suggested to be produced in NK-cells and ILC1 in order to activate and potentiate macrophages (23, 24). A recent study also identifies IFNG as an intestinal vessel disruptor and implies it in IBD pathogenesis (23). In a previously published paper no difference in *IFN*G expression was found between UC remission patients and healthy controls (14).

Relatively low expression of IL33 was protective in our data and in previous literature it has been proposed to have dualistic properties(25, 26). It is found to be upregulated in inflamed mucosa especially in epithelial cells (27, 28), and it is believed to function as an “alarmin” that rallies the colonic immune-defence (29). In contrast, IL33 is thought to ameliorate inflammation through macrophage modulation (30), and it is found to be up regulated in remission compared to healthy individuals (14). Immunostaining showed no difference in IL33 amount between UC remission and normal controls. IL33 positive cells were in the lamina propria, identified as endothelial cells or mononuclear cells, this is in line with previous report (27). IL33 in the lamina propria could represent an ongoing healing process, as IL 33 and its receptor ST2 has been linked to wound healing and fibrosis (31, 32).

If validated this IFNG:IL33 ratio may server as an early detection warning for patient in remission as the median time for relapse for the low ratio group was 7.5 months while the high ratio group never reached median time during the follow up. The ratio achieved an adequate sensitivity of 57.1%, but with high specificity of 92.6%. Despite the adequate sensitivity the positive predictive value was 80.0% with a negative predicting values of 80.1% it is indicating that a patient in the low ratio is likely to experience a relapse. In the clinical setting where the ratio could aid the clinical judgement the

high specificity would prevent unnecessary intensifying of treatment, which in turn gives better patient care.

In this study several clinical factors where investigated and none of the following factors showed association with relapse: UCCS, MES and histology. UCCS and MES showed potential as they had non-zero coefficients but ultimately failed to reach significance. This is likely due to a lack of power as lager studies have found it beneficial (33, 34). In the case of histology, we have previously reported that there is substantial intra-rater variation in histologic evaluation which could mask any predictive ability of histologic evaluation (12).

The strength of this study was the method of detection for the biomarker and the representativeness of the participants for the UC remission population. The method for finding the potential biomarkers is broad and unbiased which reduce the chance of falsely disregarding results. The participant group was heterogeneous in relation to medication, disease duration and age, which made the results applicable to a real-world setting.

The study was challenged by low number of cases as overfitting was a concern and few variables could be included in the final model. With a higher number of participants, a better model could possibly have been found which might improve the sensitivity and specificity. Furthermore, the present study was exploratory only and therefore these findings need to be validated in a second dataset, and the usefulness must be further explored in a prospective intervention study. Immunostaining on IFNG is a challenge due to low specificity of IFNG-antibodies. This is previously reported and the human protein atlas only rate it as “approved” under immunohistochemistry data reliability (35-37).

In conclusion, the ratio between IFNG and IL33 gene expression is a new and promising biomarker for predicting disease relapse in UC patients in remission, independent of medication. Further studies are needed to validate this finding, and the mechanisms behind this ratio may have interesting implication to the pathophysiology of UC relapse.

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Supplement 1

Table 3 Result from forward selection. Suggesting an model based on IL33, IFNG and MES.

Table A Cox PH fit			
Variable	Beta (SE)	HR (95% CI)	P
IL33	1.006 (0.31)	2.73 (1.50, 4.97)	<0.001
IFNG	-0.49 (0.25)	0.61 (0.38, 0.99)	0.045
MES	1.20 (0.62)	3.31 (0.98, 11.27)	0.054

Figure 3 KM comparison over the some feature used evaluation of the remission state. The only significant is IFNG:IL33 ration, but UCCS shows tendencies suggesting that it could be significant in a bigger data set.

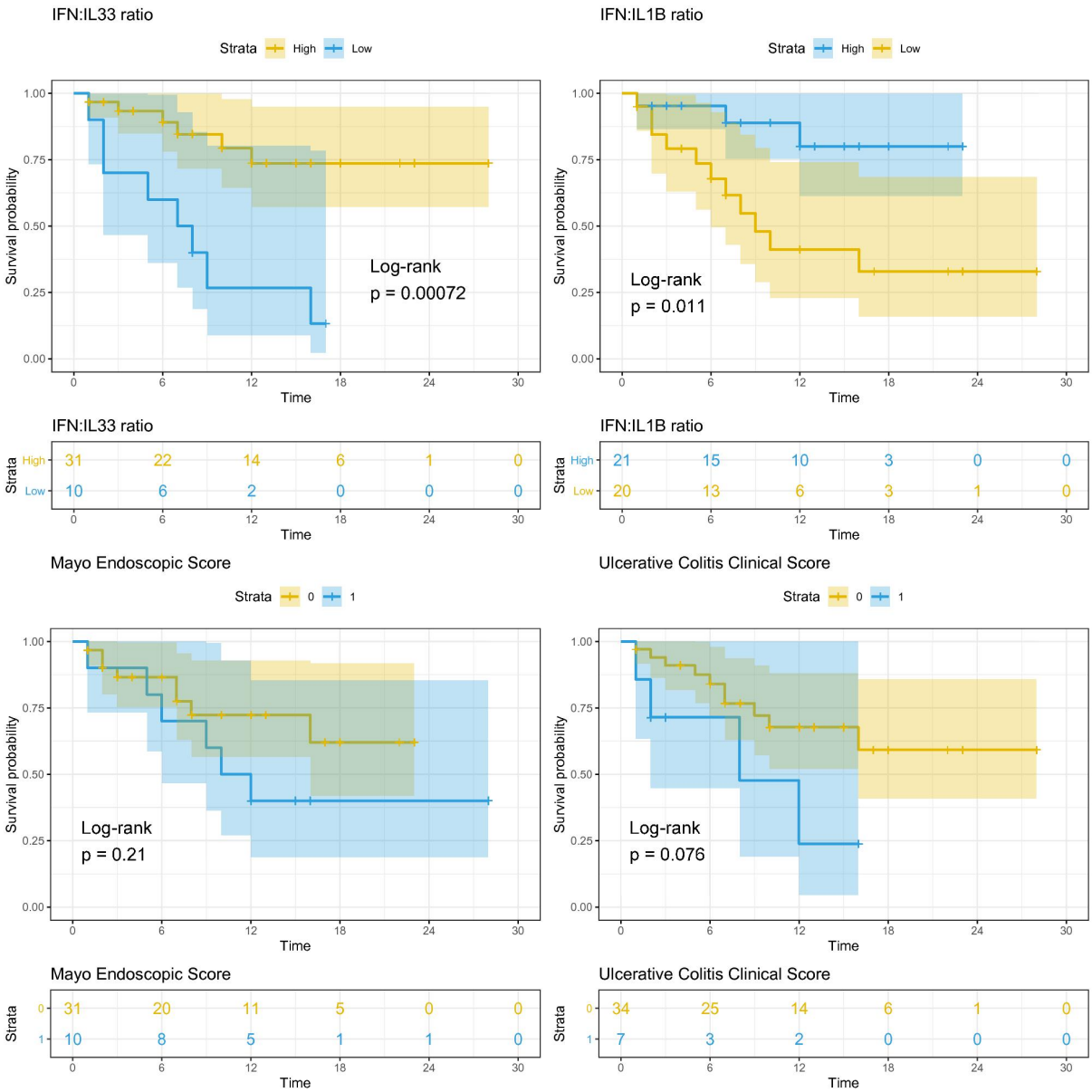

Figure 4 Correlation between IL33 and IL1RL1

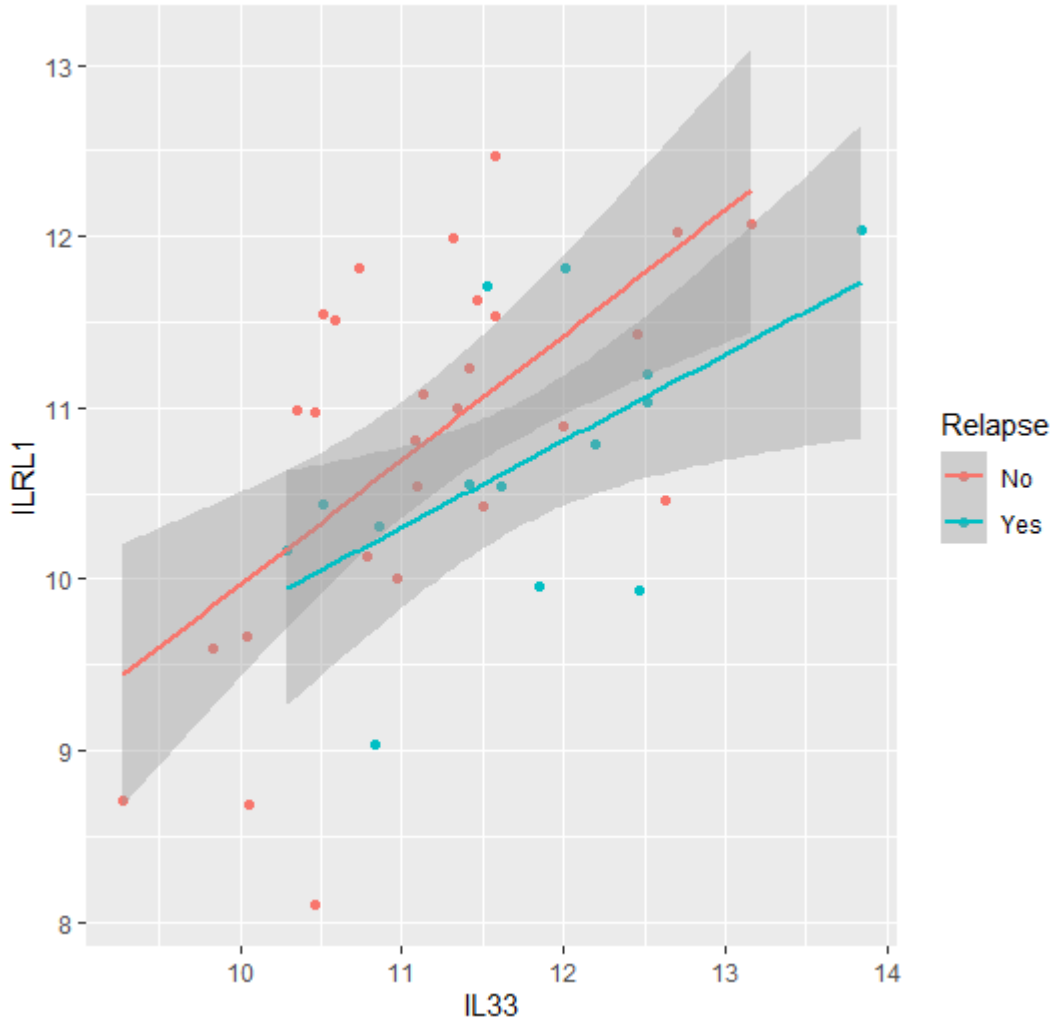

Table 4 All covariates tested with the LASSO regreission

Clinical	Pathological	Genetical
Biopsy location	Robarts Histopathological Index	IL1B
Sex	Geboes score	IL6
Age	Nancy Index	IL8
Disease duration	GS Remission Strict	IL10
Anti-TNF treatment	RHI Remission Strict	IL18
MES	Nancy Remission Strict	IL21
UCCS	GS Remission Relaxed	IL23
Biological medication	RHI Remission Relaxed	Il33
Steroids	Nancy Remission Relaxed	TNF
Mesalazin		TGFb
Azathioprine		IFNG
Methotrexate		TLR4
		ST2
		SPI1
		TB21
		FOXp3
		GATA3
		RORC
		ACTB
		IL17
		IL4

Table 5 Genes with the corresponding primers, probe and tested efficiency.

Gene	FP	RP	Probe	Efficiency
IL6	CCA-GGA-GCC-CAG-CTA-TGA-AC	CCC-AGG-GAG-AAG-GCA-ACT-G	CCT-TCT-CCA-CAA-GCG-CCT-TCG-GT	104%
FOXP3	GAG-AGG-CTG-AGT-GCC-ATG-CA	GGA-GCC-CTT-GTC-GGA-TGA-T	AAT-GGC-ACT-GAC-CAA-GGC-TTC-ATC-TGT-G	97%
IL33	TGA-GTC-TCA-ACA-CCC-CTG-AAA-TG	GGC-ATG-CAA-CCA-GAA-GTC-TTT-	CAG-GTG-ACG-GTG-TTG-ATG-GTA-AGA-TGT-TAA-TG	96%
IL18	GGC-CTC-TAT-TTG-AAG-ATA-TGA-CTG-ATT	CCA-TAC-CTC-TAG-GCT-GGC-TGG-CTA-TCT-TTT	TGA-CTG-TAG-AGA-TAA-TGC-ACC-CCG-GAC-CAT-ATT-TAT-TAT-A	100%
TLR4	CCT-TCT-CAA-CCA-AGA-ACC	CTG-GAT-TTC-ACA-CCT-GGA-TA	CCC-TGA-GGC-ATT-TAG-GCA-GC	100%

TBX21	GGC-GTC-CAA-CAA-TGT-GAC-C	CAA-CGA-TAT-GCA-GCC-GGG	AGA-TGA-TTG-TGC-TCC-AGT-CCC	96%
TGFB	CTG-CTG-AGG-CTC-AAG-TTA-AAA-GTG	TGA-GGT-ATC-GCC-AGG-AAT-TGT	CAG-CAC-GTG-GAG-CTG-TAC-CAG-AAA-TAC-AGC	100%
IL21	TAT-GTG-AAT-GAC-TTG-GTC-CCT-GA	AGG-AAA-AAG-CTG-ACC-ACT-CAC-AG	TTT-CTG-CCA-GCT-CCA-GAA-GAT-GTA-GAG-ACA-A	90%
IL23	CCC-AAG-GAC-TCA-GGG-ACA-AC	TCC-TAG-CAG-CTT-CTC-ATA-AAA-AAT-CA	TCA-GTT-CTG-CTT-GCA-AAG-GAT-CCA-CCA-G	97%
IL17	TGA-TTG-GAA-GAA-ACA-ACG-ATG-ACT	ATT-GTG-ATT-CCT-GCC-TTC-ACT-ATG	TGG-TGT-CAC-TGC-TAC-TGC-TGC-TGA-GC	100%
SPI1	CCA-TCA-GAA-GAC-CTG-GTG-	CCA-GTA-ATG-GTC-GCT-ATG	ACA-CGG-TAC-TAT-ACC-AAC-GCC-AA	93%
TNF	CAC-GCT-CTT-CTG-CCT-GCT-G	GAT-GAT-CTG-ACT-GCC-TGG-GC	CCA-GAG-GGA-AGA-GTT-CCC-CAG-GGA-C	111%
ACTB	TGC-CGA-CAG-GAT-GCA-GAA-G	GCC-GAT-CCA-CAC-GGA-GTA-CT	AGA-TCA-AGA-TCA-TTG-CTC-CTC-CTG-AGC-GC	97%
IL10	CGA-GAT-GCC-TTC-AGC-AGA-GTG-	TCA-TCT-CAG-ACA-AGG-CTT-GGC	CCT-TGC-TGG-AGG-ACT-TTA-AGG-GTT-ACC-TGG	106%
RORC	CAG-CGC-TCC-ACC-ATC-TTC-C	GCA-CAC-CGT-TCC-CAC-ATC-TC	AGG-AAG-TGA-CTG-GCT-ACC-AGA-GGA-AGT-CCA-T	108%
IL1B	CCT-GAG-CTC-GCC-AGT-GAA-A	TTT-AGG-GCC-ATC-AGC-TTC-AAA	ATG-GCT-TAT-TAC-AGT-GGC-AAT-GAG-GAT-GAC-T	98%
IL4	CGG-CTC-GAC-AGG-AAC-CTC-T	TCC-AAG-TTT-TCC-AAC-GTA-CTC-T	CGG-GCT-TGA-ATT-CCT-GTC-CTG-TGA-AG	102%
IFNG	AGC-GGA-TAA-TGG-AAC-TCT-TTT-CTT-AG	AAG-TTT-GAA-GTA-AAA-GGA-GAC-AAT-TTG-G	CAT-TTT-GAA-TTG-GAA-AGA-GGA-GAG-TGA-C	107%
IL1RL1	ACA-CCT-GTA-AAT-TTA-TAC-ACA-ATG-A	CCT-TGC-TCA-TCC-TTG-ACC	CCT-GGT-CGC-CGT-CAC-ACT-AT	96%
GATA3	GCG-AGC-AAC-GCA-ATC-TGA	GGG-CGA-CGA-CTC-TGC-AAT	AGG-TGA-CCC-GAG-GAG-GGA-CTC-CG	97%

IL8	TVT-TGG-CAG-CCT-TCC-TGA-TT	TTT-CTG-TGT-TGG-CGC-AGT-GT	CTG-CAG-CTC-TGT-GTG-AAG-GTG-CAG-T	104%

Supplement 2

Packages

Forward selection	MASS v7.3-51.6
LASSO regression	glmnet v4.0-2
Survival analysis	survminer v0.4.8, survival v3.2-3
ROC analysis	pROC v1.16.2
Impute	Impute 1.58