UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Division of Infection, Inflammation and Immunity

The role of microRNAs miR-31 and miR-155 in Ulcerative Colitis

MiR-31 and miR-155 counteract the dysregulation of the IL-13 pathway in Ulcerative Colitis. Effects of IBD treatment on gene expression.

by

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ABSTRACT

Ulcerative colitis (UC) is an inflammatory disease of the colonic mucosa driven by a Th2-like response in which IL-13 leads to toxic effects to the mucosa and activation of the IL-13/STAT6 pathway. IL-13 signalling depends on the expression of the receptor IL13Rα1 and *in silico* analysis predicted that miR-31 directly targets the 3' UnTranslated Region (3'UTR) of IL13Rα1 mRNA. MicroRNAs are small non-coding RNAs that inhibit gene expression by pairing to the 3'UTR of their target mRNAs. MiR-155 directly targets IL13Rα1 and together with miR-31 has been shown to be up-regulated in active UC. Both microRNAs are involved in the regulation of innate and adaptive immune responses. My hypothesis was that up-regulation of miR-31 and miR-155 reduces IL13Rα1 expression in an attempt to mitigate the over-activated IL-13/STAT6 pathway that contributes to inflammation in UC. Our analysis showed that IL-13 dependant genes were significantly up-regulated while IL13Rα1 mRNA and protein expression was found significantly down-regulated in paired active UC sigmoid biopsies compared to unaffected inactive UC (both from a representative UC population) and normal controls. Inversely to IL13Rα1 expression, mir-31 and miR-155 levels were significantly elevated in active UC in the same samples. Using *in vitro* experiments, it was demonstrated that miR-31 directly targets IL13Rα1. In order to dissect the role of miR-31 and miR-155 in the IL-13/STAT6 pathway in UC, pre-microRNA transfections in different cellular systems were used. HT-29 cells provided evidence of a role for miR-31 and miR-155 in colonic epithelial cells in the down regulation of IL13Rα1 and STAT6 activation. Similar effects were observed in THP-1 monocytic cells and primary human macrophages which were used as models of lamina propria macrophages. Importantly, pre-microRNA transfections of biopsy explant cultures from patients with active UC showed that miR-31 and miR-155 can down-regulate the levels of IL13Rα1 and IL-13-dependent genes *ex vivo*, providing their potential therapeutic application in UC. Furthermore, in an attempt to reveal a molecular signature of responders and non-responders to drug treatment in IBD, we investigated the expression of microRNAs involved in inflammation and immunity (miR-31, miR-146a and miR-155) as well as the levels of Th1-, Th2- and fibrosis/tissue remodelling-related genes. Sigmoid biopsies from treatment naïve patients with inactive and active UC and Crohn's (CD) were compared to patients undergoing treatment with 5-aminosalicylates (UC), Thiopurines (UC and CD) and anti-TNF-α agents (CD). This revealed a significantly different expression profile of these microRNAs and genes according to disease activity and treatment response. These findings may help to identify biomarkers of clinical significance for IBD therapy..

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Abbreviations

3'UTR: 3' UnTranslated Region

5-ASA: AminoSAlicylates

AZA: azathioprine

CARD9: CAspase Recruitment Domain family member 9

CCL18: Chemokine (C-C motif) Ligand 18

CD: Crohn's Disease

CDAI: Crohn's Disease Activity Index

cDNA: Copy DNA

CDH1: E-cadherin

CRC: ColoRectal Cancer

DALM: Dysplasia-Associated Lesion or Mass

DC: Dendritic Cell

DC-SIGN: Dendritic Cell -Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin

ECM: ExtraCellular Matrix

ECM1: ExtraCellular Matrix protein 1

ECCDS: European Cooperative Crohn's Disease Study

ERAP2: Endoplasmic Reticulum AminoPeptidase 2

FCS: Foetal Calf Serum

Foxp3: Forkhead box P3

GALT: Gut-Associated Lymphoid Tissue

GWAS: Genome-Wide Association Studies

HLA: Human Leukocyte Antigen

HNF4a: Hepatocyte Nuclear Factor 4 alpha

IBD: Inflammatory Bowel Disease

IDC: Indeterminate Colitis

IFN-γ: Interferon gamma

IGA, IgA: Immunoglobulin A

IL-1, 2, 4, IL-5, IL-10, IL-12, IL-13, IL-21, IL-22, IL-26, and IL-27: Interleukin 1, 2,4, 5, 10, 12, 13, 21, 22, 26 and 27 respectively

IL1R2: Interleukin 1 Receptor 2

IL2RA: Interleukin 2 Receptor alpha

IL7R: Interleukin 7 Receptor

IL8RA: Interleukin 8 Receptor A

IL12B: Interleukin 12 subunit p40

IL13Rα1: Interleukin 13 Receptor alpha 1 (mRNA or gene)

IL23R: Interleukin 23 Receptor

iNOS: inducible NO Synthase

JAK2: JAnus Kinase 2

LAMB1: Laminin beta 1

LPS: bacterial LipoPolySacharide

M cell: human Micro-fold cells

miR: microRNA

mRNA: messenger RNA

MUC2: MUCin 2

NF-κB: Nuclear Factor Kappa B

NKT: Natural Killer T cells

NLRs: Nucleotide-binding domain and Leucine-rich repeat-containing Receptors

NOD2: Nucleotide-binding Oligomerization Domain containing 2

OCP: Oral Contraceptive Pill

PRRs: Pattern Recognition Receptors

PSC: Primary Sclerosing Cholangitis

RISC: RNA-Induced Silencing Complex

ROS: Reactive Oxygen Species

RT-qPCR: Reverse Transcription followed by Quantitative PCR

SES-CD: Simple Endoscopic Score for Crohn's Disease

SMAD3: SMAD family member 3

SNP: Single Nucleotide Polymorphism

SOCS1: Suppressor of Cytokine Signalling 1

STAT1, STAT3, STAT6: Signal Transducer and Activator of Transcription 1, 3 and 6, respectively

TNF-α: Tumour Necrosis Factor alpha

T-regs: T regulatory cells

TFF3: TreFoil Factor-3

UC: Ulcerative Colitis

1. Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is comprised of two major disorders: Ulcerative colitis (UC) and Crohn's disease (CD). Up to 10% of cases of IBD cannot be classified in either category and is therefore termed IBD as yet unclassified (IBDU). They are incurable, lifetime debilitating intestinal inflammatory illnesses. CD and UC have been known to manifest a variety of extra intestinal complications that affect the joints, eyes, and skin and liver in addition to their intestinal manifestations. The main symptoms of IBD are bloody diarrhoea, abdominal pain, weight loss and the mentioned involvement of extra-intestinal complications such as joint pains, skin rashes and red eyes.

The pathogenesis of IBD is multifactorial and the development of IBD is understood to be associated with a complex interplay of genetic, bacterial and environmental factors leading to a dysregulation of the innate and adaptive immune systems. Long-standing IBD impairs the quality life of patients significantly and also increases the risk of colorectal cancer as compared to the non-IBD population.

1.1.1 Epidemiology of IBD

In general, the highest incidence and prevalence for both CD and UC has been reported in northern Europe, United Kingdom and North America, which are the geographic regions that have been historically associated with IBD. In Europe, incidence rates for inflammatory bowel disease range from 1.5 to 20.3 cases per 100,000 person-years for UC and from 0.7 to 9.8 cases per 100,000 person-years for CD (Loftus 2004).

In North America, incidence rates for IBD range from 2.2 to 14.3 cases per 100,000 person-years for UC and from 3.1 to 14.6 cases per 100,000 person years for CD. Prevalence ranges from 37 to 246 cases per 100,000 persons for UC and from 26 to 199 cases per 100,000 persons in the case of CD (Loftus 2004).

A study from Kappelman *et al.* conducted in 33 states of USA found a prevalence of CD and UC in children younger than 20 years of 43 and 28 cases per 100,000, respectively. In adults, the prevalence of CD and UC was 201 and 238 cases per 100,000, respectively (Kappelman, Rifas-Shiman et al. 2007).

CD and UC are most commonly diagnosed in late adolescence and early adulthood, but the diagnosis may occur at all ages. Mean and median ages at diagnosis of UC are in general 5 to 10 years later than those associated with CD (Loftus, Schoenfeld et al. 2002). The peak age for CD occurrence is between 20 to 30 years and UC is most commonly diagnosed at the ages between 30 and 40 years. In a systematic review of population-based cohorts of CD from North America, the mean age at diagnosis ranged from 33.4 years to 45 years (Loftus, Silverstein et al. 2000). A further peak has of IBD occurrence has been reported between 60 and 70years of age (Loftus 2004).

IBD in childhood accounts for 7% to 20% of all IBD cases which is based on the varying results from population-based studies (Auvin, Molinie et al. 2005, Jacobsen, Fallingborg et al. 2006, Kelsen and Baldassano 2008).

Lenard-Jones *et al* found in a European study incorporating 20 countries from Iceland to Israel a male predominance of UC with male versus female ratios of 1.2 and 1.5. In CD more women than men were found to be affected, showing a male versus female ratio 0.8 in the northern centres and 1.0 in those in the south (Lennard-Jones and Shivananda 1997). Several regional differences in the UC and CD distribution have been noted both geographically and in the male versus female ratio of the diseases (Lennard-Jones and Shivananda 1997). However in summery, the overall distribution of genders in CD shows a slightly higher prevalence in women and in UC men are slightly more commonly affected than women.

Certain distinct ethnic groups such as the Jewish population, especially the Ashkenazi Jews, have a 2-4 times increased incidence to develop IBD when compared to non-Jewish Caucasians. American Caucasians and African Americans have a higher risk of IBD compared to the Hispanic and Asian population (Roth, Petersen et al. 1989, Nguyen, Torres et al. 2006).

Immigration from countries with a low prevalence of IBD to regions of higher prevalence have shown that these individuals have an increased risk of acquiring IBD, especially first generation offspring (Bernstein and Shanahan 2008, Mikhailov and Furner 2009). This suggests that western lifestyle may well play a part in the development of the IBD.

1.1.2 Aetiology of IBD

1.1.2.1 Introduction

Theories regarding the pathogenetic mechanisms causing IBD are multifactorial and thought to be a combination of epidemiological factors, the gut/environmental interface and the inflammatory process triggered by a mucosal immunity imbalance and genetics of each disease. The exact aetiology of IBD remains unknown. It is hypothesized that IBD affects genetically susceptible individuals who respond to environmental triggers such as the intestinal gut flora, infections, drugs, smoking or food, in order to develop a disease such as CD and UC. The genetic component is believed to be stronger in CD than in UC (Blumberg and Strober 2001).

In addition to the absorption of nutrients, the intestinal mucosa has to fulfil a variety of tasks. This invoves the provision of an effective epithelial barrier, signal transduction and sensing of potentially harmful luminal contents. The intestinal mucosa is exposed to a variety of endogenous and exogenous stimuli. Endogenous antigens consist of a wide spectrum of microbial species and their products as part of the intestinal bacterial flora. Exogenous antigens comprise nutrients and exogenous bacterial stimuli. In order to keep a healthy balance, the sensing through surface receptors triggers an appropriate response of the mucosal immune system in the lamina propria either tolerating the stimulus or eliminating it. It therefore provides defence against enteric pathogens and on the other hand keeps a functional intestinal equilibrium through balanced mucosal immunity and tolerance in the healthy gut (Chichlowski and Hale 2008).

Dysregulation of the mucosal immune system has been implicated in the pathogenesis of IBD. Losing the tolerance to commensal bacterial gut flora or defects in mucosal barrier function can trigger an inappropriate response through activation of the innate and adaptive immune systems (Loftus 2004). This immune activation results in the synthesis and release of pro-inflammatory cytokines triggering a cascade that leads to intestinal inflammation which results in loss of mucosal barrier function and perpetuation of inflammation, tipping the balance of the intestinal equilibrium (Stallmach, Strober et al. 1998, Podolsky 2002). Genes involved in sensing the intra-luminal intestinal content such as NOD2 (Hugot, Chamaillard et al. 2001) and CARD9 (Anderson, Boucher et al. 2011) or genes implicated in mucosal barrier function such as ECM1,

CDH1 and HNF4a (Khor, Gardet et al. 2011) have been found to be defective in a significant number of IBD patients.

1.1.2.2 Environmental aspects of inflammatory bowel disease

Smoking seems to be protective against UC as it mainly affects non-smoker and ex-smokers, but worsens the course of CD considerably. Smoking in UC lowers the number of flare ups and decreases the likelihood of colectomy, but makes escalation of treatment, complications of the disease and surgery more likely in CD. The mechanism of these diverse effects is unknown (Calkins 1989).

A population-based cohort study performed with 771 UC patients prospectively included and followed for 10 years, revealed a lower relapse rate in smokers compared with non-smokers (Höie, Wolters et al. 2007). A study carried out in the Netherlands by van der Heide et al with 295 UC patients identified smoking after diagnosis as a protective factor for colectomy (van der Heide, Wassenaar et al. 2011).

Some authors still propose “mild smoking” as an alternative extreme therapy in patients with therapy resistant UC (Cottone, Georgios et al. 2011).

On the contrary, in Crohn’s disease smoking has been associated with a higher risk of relapse (Holdstock, Savage et al. 1984) and increased need for immunomodulators (Cosnes, Carbonnel et al. 1996). Benefits of smoking cessation have been demonstrated in a study by Cosnes et al showing that patients who stop smoking for at least 6 months are at a lower risk of relapse in the following 12-18 months, as compared to non-quitters (Cosnes, Beaugerie et al. 2001).

While the natural history of colonic CD seems to be the same in smokers and non-smokers (Cosnes, Carbonnel et al. 1999), the rates of relapse and intestinal resection are higher among smokers with ileal disease (Holdstock, Savage et al. 1984). In addition, smoking has been associated with earlier progression to stricturing and penetrating disease behaviour, thus suggesting that tobacco facilitates progression towards complicated disease (Louis, Michel et al. 2003).

Postsurgical recurrence is the clinical scenario in which active smoking has been most conclusively proven to worsen CD prognosis both in the short- and long-term (Reese, Nanidis et al. 2008).

Appendectomy has been shown to lower risk of suffering from UC, particularly in children who are operated before the age of 10, as shown in a meta-analysis of 17 case-control studies (Koutroubakis and Vlachonikolis 2000). The link between appendectomy and CD is less conclusive and a meta-analysis showed an increased risk of CD in patients who underwent appendectomy. The association was particularly strong for those appendectomies performed within one year before CD diagnosis, and less conclusive for those performed five years prior to the diagnosis of CD (Kaplan, Jackson et al. 2008).

The hygiene theory postulates that with the improvement of sanitation and living standards the exposure to pathogens in childhood has decreased, therefore explaining the increased frequency of IBD in cities as a result (Klement, Lysy et al. 2008).

There is a clear inverse correlation between the frequency of helminth infections and the prevalence of IBD which potentially explains the north south divide of IBD, as helminth infections are much more common in warmer climates. Reports have now appeared showing IBD control after natural exposure to intestinal helminths (Büning, Homann et al. 2008).

There have been several clinical trials using helminths to treat IBD. Results from these trials suggest that infection with helminths improves clinical outcome, which supports the theory that natural helminth infection is protective. There was clinical improvement in a double-blind clinical study in UC and an open-label study in CD. These studies used live ova from porcine whipworm (Trichuris suis) as an oral therapeutic intervention (Summers, Elliott et al. 2005, Summers, Elliott et al. 2005).

Western diet has also been implicated in the causation of IBD (Powell, Harvey et al. 2000); it relates to the higher incidence of IBD in the first generation of immigrant population from countries with a low prevalence of IBD to high prevalence areas of IBD (Bernstein and Shanahan 2008, Mikhailov and Furner 2009).

The effect of oral contraceptives on the risk of IBD appears to be related to estrogens. Estrogen acts as an immune enhancer and may increase the production of tumor necrosis factor by macrophages. The oral contraceptive pill (OCP) has been linked with an increased risk of IBD (Cornish, Tan et al. 2008).

Non-steroidal anti-inflammatory drugs cause ulceration throughout the gastrointestinal tract and have been shown to increase the risk of IBD (Felder, Korelitz et al. 2000). Several mechanisms for this phenomenon have been suggested such as cycloxygenase (COX) inhibition, leukotriene shunting or inhibition of NF-κB activity, although none of them has been conclusively demonstrated. Most studies evaluating the impact of NSAID use on IBD relapse agree on the potential deleterious effect of these drugs on quiescent IBD (Feagins and Cryer 2010), (Kefalakes, Stylianides et al. 2009). In the only prospective controlled study assessing disease relapse with the use of different NSAIDs as compared to acetaminophen in IBD patients without arthritic complaints, a significantly increased risk of relapse with NSAIDs was reported (Takeuchi, Smale et al. 2006). It is also still debated whether selective COX-2 inhibitors are safer than conventional NSAIDs for patients with IBD. A prospective, randomized, double-blind, controlled trial performed to date showed no increase in UC flares as compared to placebo (Sandborn, Stenson et al. 2006).

1.1.2.3 Genetics of IBD

Familial clustering raised the suspicion of a genetic component to IBD and was subsequently confirmed in twin studies, showing increased concordance rates for UC and CD in monozygotic twins as compared with dizygotic counterparts (Orholm, Binder et al. 2000). Genetic factors seem to be somewhat less significant for UC than CD (10% as compared with 37% concordance for monozygotic twins). The relative risk of developing UC in a sibling is 8–15 and 25–42 for CD (Russell and Satsangi 2004).

Understanding of the genetic components underlying IBD has been hindered by the complex nature of its pathology. Genome-Wide Association Studies (GWAS) have proven a powerful tool to start to untangle the different aspects of IBD. GWAS provide a genome-wide picture of the different genetic variants, mostly Single Nucleotide Polymorphisms (SNPs) and genetic loci, associated to a certain phenotype (CD, UC or both). Genetic loci identified in different GWAS represent a wide host of functions implicated in the pathogenesis of IBD such as intestinal

homeostasis, epithelial barrier integrity and restitution, microbial sensing and defence, regulation of innate and adaptive immunity, autophagy and metabolic cellular pathways (Khor, Gardet et al. 2011). An overview of IBD susceptibility genes identified to date is presented in the diagram in ***Figure 1*** (Thompson and Lees 2011). So far 99 non-overlapping genetic risk loci have been identified, including 28 that are shared between CD and UC (Franke, McGovern et al. 2010, Anderson, Boucher et al. 2011).

NOD2 (Nucleotide-binding Oligomerization Domain containing 2) is the most significant CD susceptibility gene to date. Discovered and associated for the first time to CD in 2001 (McGovern, van Heel et al. 2001, Ogura, Bonen et al. 2001). NOD2 is a Pattern Recognition Receptor (PRR) that acts as sensor recognising intestinal bacterial wall products invading the epithelial barrier and may trigger mucosal defence mechanisms, but its exact mechanism is unclear. NOD2 is thought to induce autophagy in dendritic cells modulating antigen presentation and bacterial processing (Cooney, Baker et al. 2010). A defective NOD2 gene therefore may predispose to CD (Hampe, Franke et al. 2007, Cooney, Baker et al. 2010). Hampe *et al.* described a SNP associated with CD in the ATG16L1 gene, which encodes a protein that is part of the phagosomal system for intracellular bacterial processing and its role as Crohn's disease susceptibility gene was confirmed by Cummings et al. (Cummings, Cooney et al. 2007, Hampe, Franke et al. 2007). Several GWAS have identified CD-associated variants (Yamazaki, McGovern et al. 2005) and recently Franke *et al* have identified 30 new susceptibility loci meeting genome-wide significance for CD including functionally interesting candidate genes such as SMAD3, ERAP2, IL-10 and IL2RA bringing the total number of susceptibility genes to 71 in CD (Franke, McGovern et al. 2010).

Due to the lower heritability of UC, less associated loci have been identified prior to the use of GWAS. UC genes of significance include HLA DRB1*0103 allele (Lappalainen, Halme et al. 2008), a rare variant at the HLA class II region which relates to the phenotype of disease showing increased association with severity of the disease (Thompson and Lees 2011). IL-10 has been identified as a sole susceptibility locus for UC. IL-10 knock out animal models have demonstrated the importance of IL 10 on colitis as a regulator of B and T-cells and also has an inhibitory effect on pro-inflammatory cytokines such as TNF-α and IL-12. Further UC susceptibility genes of note are IL-2, IL-21, IL-22, IL-26 and IL-27, as well as genes associated with epithelial barrier such as ECM1, CDH1, HNF4a, and LAMB1 regulating mucosal permeability

(Thompson and Lees 2010). Further UC genes include interferon gamma (IFN-γ) and CARD9 which are both involved in the inflammatory response (Thompson and Lees 2011). Anderson *et al* found 29 additional UC risk loci including IL1R2, IL8RA, IL7R, IL12B and JAK2, increasing the total number confirmed associations in UC to 47 up to date (Anderson, Boucher et al. 2011). There are various examples of genetic loci associated to both UC and CD, suggestive of both pathologies also sharing common underlying pathways. The IL23R (Interleukin 23 Receptor) gene was identified as an IBD locus (Duerr, Taylor et al. 2006) and it is a shared risk between CD and UC. Other members of the IL-23 signalling pathway like IL12B, JAK2, and STAT3 have subsequently been associated with CD and then UC indicating importance for both diseases (Franke, Balschun et al. 2008).

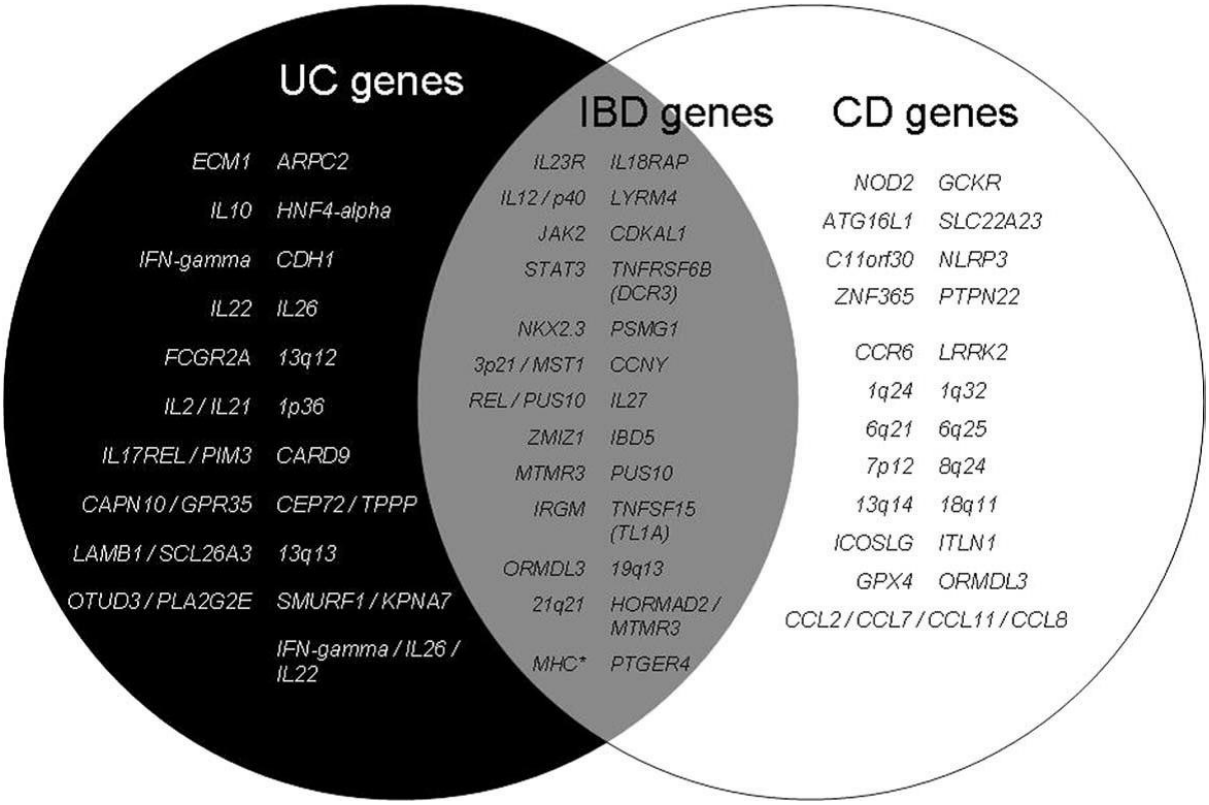


Figure 1. Overview of IBD susceptibility genes identified to date. Adapted from (Thompson and Lees 2011).

1.2 Clinical aspects of IBD

This section will discuss the clinical aspects of CD and UC focussing on the clinical differences between the two diseases, diagnosis, assessment of disease activity, the natural history of the diseases and treatment.

1.2.1 Crohn's Disease

1.2.1.1 Introduction

CD is a relapsing, trans-mural (affecting the full thickness of the gut wall) inflammatory disease that can affect any part of the gastrointestinal tract from the mouth to the anus. CD classically presents with discontinuous inflammation (skip lesions) of the gastrointestinal tract. This can lead to complications comprising of strictures, abscesses or fistulas (an abnormal connection of two body cavities or a body cavity to the skin). Extraintestinal manifestations of CD include skin, joint, eye and liver involvement. The risk of developing colorectal cancer in extensive Crohn's colitis is increased.

1.2.1.2 Clinical presentation

The clinical presentation is largely dependent on disease location and can include diarrhoea, as well as passage of blood or mucus, abdominal pain, fever, weight loss, clinical signs of mainly small bowel obstruction and extra-intestinal manifestations involving inflammatory conditions of skin, joints and eyes.

1.2.2 Diagnosis of Crohn's disease

Diagnosis of CD relies on a careful medical history inquiring about symptoms of CD such as diarrhoea (especially nocturnal), passage of blood and mucous, abdominal pain, obstructive symptoms, weight loss, evidence of perianal disease and extra-intestinal symptoms. A careful drug history, especially recent Non-Steroidal Anti-Inflammatory Drugs (NSAID) use, a detailed

travel history, recent intestinal infections, smoking status and family history of IBD should be noted.

Physical examination consists of charting heart rate, blood pressure, BMI, abdominal and perianal examination (for perianal disease) including a digital rectal examination. Eyes, skin and joints need to be examined to look for extra-intestinal features of CD.

Laboratory studies include renal function, liver function tests, full blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), iron status, B12, folic acid and possibly faecal calprotectin. Stool for microscopy, culture and sensitivities needs to be sent to exclude intestinal infection.

Colonoscopy with ileal intubation and mapping biopsies looking for inflammatory cell infiltrates with lymphocytes and plasma cells and independent granulomas should be performed. Capsule endoscopy can be helpful to assess small bowel disease.

Imagining studies commonly used for the assessment of CD include CT and MRI, especially if there is a suspicion of extra-intestinal complications such as abscesses, peri-anal and fistulating disease (MRI) or if endoscopic assessment of the ileum was unsuccessful. If primary sclerosing cholangitis is suspected magnetic resonance cholangiopancreatography (MRCP) is indicated.

High quality care with good patient outcomes is dependent on close collaboration and early involvement of other specialties including surgery, radiology, pathology, rheumatology, dermatology, hepatology, urology and gynaecology in diagnosis and management of complications and management of extra-intestinal manifestations of CD is vital (Van Assche, Dignass et al. 2010)

1.2.2.1 Clinical assessment of CD

Accurate clinical assessment and phenotyping of CD disease at diagnosis is vital for counselling the patient regarding the expected disease course, assessing the disease prognosis and is also helpful to choose the appropriate treatment for each subtype of CD early in the disease. The Montreal Classification for Crohn's disease is a tool to subtype the various phenotypes of CD and has been applied in epidemiological studies to characterise CD populations. Disease is classified according to age of onset (A1 to A3), location of disease (L1 to L4), and disease behaviour (B1-B3 with a perianal disease modifier)(Silverberg, Satsangi et al. 2005).
Table 1 shows the Montreal Classification for Crohn's disease.

Montreal classification for Crohn's disease

Age at diagnosis	**Location**	**Behaviour**
A1 below 16 years	**L1** ileal	**B1** non-stricturing, non-penetrating
A2 between 17 and 40 years	**L2** colonic	**B2** stricturing
A3 above 40 y	**L3** ileocolonic	**B3** penetrating
-	**L4** isolated upper disease*	**p** perianal disease modifier†

Table 1. Montreal Classification of Crohn's disease. *L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present. †"p" is added to B1–B3 when concomitant perianal disease is present.Adapated from (Silverberg, Satsangi et al. 2005).

Several non-invasive tools for the assessment of the activity of CD have been developed, with the Harvey-Bradshaw index (Harvey and Bradshaw 1980) and the Crohn's disease activity index (CDIA) being most frequently used in the clinical setting.

The Harvey-Bradshaw index (Harvey and Bradshaw 1980) is a simple, widely employed tool used to assess the disease activity of CD. ***Table 2*** shows the components of the Harvey-Bradshaw index (Harvey and Bradshaw 1980). Patients with CD who score 3 or less on the Harvey-Bradshaw index are considered to be in remission and patients with a score of 8-9 or higher are considered to have moderate-severe disease.

The Harvey-Bradshaw index

Variable	0	1	2	3	4
General well being	Very well	Slightly below average	Poor	Very poor	Terrible
Abdominal pain	None	Mild	Moderate	Severe	
Number of liquid stools per day					
Abdominal mass	None	Dubious	Definite	Tender	
Complications with one point for each	Arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure, new fistula, abscess				

Table 2 Harvey-Bradshaw index for assessing the clinical activity of CD (Harvey and Bradshaw 1980)

The Crohn's disease activity index (CDAI) was developed and validated in the late 1970s as part of the National Crohn's Disease Co-operative Study (Best, Becktel et al. 1976) and is mainly used in clinical research studies. Eight variables are contributing to the index such as number of loose stools, abdominal pain, well-being and haematocrit. The CDAI has been modified in several ways since its establishment and the recently developed short CDAI (sCDAI) has been shown to correlate well with the full index (Thia, Faubion et al. 2011) and is much easier to use in everyday clinical practice.

The Harvey-Bradshaw index and the CDAI do not include an endoscopic assessment of the disease activity in CD. The Simple Endoscopic Score for Crohn's Disease (SES-CD) (Daperno, D'Haens et al. 2004, Baldys-Waligorska, Kusnierz-Cabala et al. 2006) is used to assess the endoscopic appearances of CD. The SES-CD scoring system is applied to the various parts of the colon and ileum such as rectum, left colon, transverse colon, right colon and terminal ileum. The ulcer size, ulcerated and affected surface as well as stricture formation is taken into account. All five sections are added up to reach the total score. ***Table 3*** shows the definitions of Simple Endoscopic Score for Crohn's Disease (Daperno, D'Haens et al. 2004, Baldys-Waligorska, Kusnierz-Cabala et al. 2006).

Simple Endoscopic Score for Crohn's Disease values

Variable	0	1	2	3
Size of ulcers	None	Apthous ulcers (Ø 0.1 to 0.5 cm)	Large ulcers (Ø 0.5 to 2 cm)	Very large ulcers (Ø >2 cm)
Ulcerated surface	None	<10%	10-30%	>30%
Affected surface	Unaffected segment	<50%	50-75%	>75%
Presence of narrowings	None	Single, can be passed	Multiple, can be passed	Cannot be passed

Table 3 Definitions of Simple Endoscopic Score for Crohn's Disease (Daperno, D'Haens et al. 2004)

1.2.2.2 Natural history of Crohn's Disease

The peak age for CD occurrence is between 20 to 30 years of age (Loftus, Silverstein et al. 2000), although 25% of cases are diagnosed under the age of 16 years old. Despite the anatomical location of CD being fairly stable, behaviour of the disease evolves through the disease course.

At diagnosis, the disease is located in the terminal ileum in 47%, the colon in 28%, the ileo-colon in 21%, and the upper gastrointestinal tract in 3%. Disease behaviour is classified as non-stricturing and non-penetrating in 70% of patients, stricturing in 17%, and penetrating (fistulas or abscesses or both) in 13% of all patients at diagnosis (Louis, Collard et al. 2001).

Very similar results were reported in a recent population based study (patients were classified according to the Montreal classification) where more than half of over 300 patients were diagnosed between the ages of 17 and 40 years (Montreal category A2). Crohn's disease was located in the terminal ileum in 45% (L1), colon in 32% (L2), ileocolon in 19% (L3), and upper gastrointestinal tract in 4% (L4). The majority of patients (81%) had a non-stricturing non-penetrating phenotype (B1), 5% a stricturing (B2) and 14% a penetrating (B3 or B3p) phenotype (Thia, Sandborn et al. 2010).

The most prominent change is from non-stricturing inflammatory disease to either stricturing (in 27%) or penetrating (in 29%) disease (Louis, Collard et al. 2001). One year after diagnosis, 10–30% of patients with CD have active disease, 15–25% have low activity, and 55–65% are in remission. 13–20% of patients with CD have a chronic active course of disease activity, 67–73% have a chronic intermittent course and only 10–13% remain in remission for several years (Munkholm, Langholz et al. 1995, Loftus, Schoenfeld et al. 2002). In the Thia et el study 19% of patients progressed to a more aggressive phenotype within 3 months and more than half (51%) at 20 years after initial diagnosis, especially when ileal and perianal involvement (fistulas) were present at the time of diagnosis (Thia, Sandborn et al. 2010).

After 20 years, most patients with CD will require surgery (Cosnes, Cattan et al. 2002). The life expectancy of patients with CD is slightly reduced (Jess, Winther et al. 2002). There is an 18-fold increase in the risk of developing colorectal cancer in extensive Crohn's colitis after 20 years disease duration as compared to the general public (Gillen, Walmsley et al. 1994).

1.2.3 Ulcerative Colitis

1.2.3.1 Introduction

UC is a relapsing non-trans-mural inflammatory disease that is restricted to the colon. Dependent on the anatomic extent of involvement, patients can be classified as having proctitis (rectum only), left-sided colitis (extending to the sigmoid colon with or without involvement of the descending colon), or pan-colitis extending the whole length of the colon. Some patients with pan-colitis also develop ileal inflammation (backwash ileitis) (Abdelrazeq, Wilson et al. 2005), which occasionally complicates differentiation between CD and ileo-colitis.

1.2.3.2 Clinical presentation

Patients typically present with bloody diarrhoea (often nocturnal and postprandial), passage of pus, mucus, or both, and abdominal cramping during bowel movements. Systemic symptoms such as malaise, anorexia, weight loss and fever indicate severe attacks of UC. Severe symptoms are less common in left-sided colitis and proctitis (Baumgart and Sandborn 2007).
Extra-intestinal features such as axial or peripheral arthropathy, episcleritis and erythema nodosum may accompany the presentation in about 10% and rarely precede intestinal symptoms (Danese, Semeraro et al. 2005) Thrombo-embolism is more common in UC as compared to the normal population especially in active disease and pan-colitis (Danese, Papa et al. 2007). Smoking seems to be protective against UC; patients can present with a history of recently giving up smoking and is smoking us associated with a less severe disease course. (Calkins 1989).

1.2.4 Diagnosis of Ulcerative Colitis

Diagnosis of UC relies on a careful medical history inquiring about onset of symptoms of UC such as recurrent rectal bleeding and diarrhoea (especially nocturnal), passage of blood and mucus, urgency, tenesmus, incontinence, abdominal pain, weight loss and extra-intestinal symptoms. A careful drug history, especially recent NSAID use, a detailed travel history, recent intestinal infections, smoking status, previous appendicectomy and family history of IBD should be noted.

Physical examination consists of charting heart rate, blood pressure, BMI, abdominal and perianal examination (including a digital rectal examination). Eyes, skin and joints need to be examined to look for extra-intestinal features of UC.

Laboratory studies include renal function, liver function tests, full blood count, erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), iron status and faecal calprotectin. Stool for microscopy, culture and sensitivities needs to be sent to exclude intestinal infection including *Clostridium difficile* and Cytomegalovirus.

Colonoscopy with ileal intubation and mapping biopsies looking for mucosal and crypt architectural distortion, mucosal atrophy and a villous or irregular mucosal surface need to be performed for diagnosis, extent of disease, disease activity and detection of dysplasia. Surveillance colonoscopies with mapping biopsies or targeted biopsies with chromoendoscopy are performed after 8-10 years of disease onset to reassess disease and plan the surveillance strategy to facilitate the early diagnosis of dysplasia or colorectal cancer.

Abdominal plain radiographs are indicated in the assessment of severe UC to look for colonic dilation, mucosal oedema and disease extent. Other imaging modalities such as Ultrasound, CT or MRI are indicated if endoscopic evaluation is not possible to determine extent of disease severity (Dignass, Eliakim et al. 2012).

1.2.4.1 Natural history of UC

UC is most commonly diagnosed at the ages between 30 and 40 years (Loftus, Silverstein et al. 2000). Half the patients are in clinical remission at any given time although 90% have an intermittent course. In the first 3–7 years after diagnosis, 25% of patients are in remission, 18% have activity every year, and 57% have intermittent relapses (Langholz, Munkholm et al. 1994). The only significant predictor of remission or relapse was disease activity in the preceding year. After 10 years, the colectomy rate was 24% (Langholz, Munkholm et al. 1994), but in later studies the colectomy rate was quoted to be much lower. More than half the patients with left-sided colitis will progress proximally during 25 years. During the same period, patients with more extensive disease regress in about 75% of cases (Langholz, Munkholm et al. 1996). Overall, patients with UC have been shown to have a normal life expectancy (Winther, Jess et al. 2003).

Solberg et al conducted a cohort study including over 400 newly diagnosed patients with UC and followed them up over 10 years. Again the mortality risk was not increased compared to the general population. 55% were in remission or had mild symptoms after high activity of disease at diagnosis. Only 1% of patients had severe symptoms after presenting with mild disease at diagnosis during the first 10 years. 6 % had a chronic continuous course over 10 years and 39% had a chronic intermittent course. The cumulative colectomy rate after 10 years was 9.8%. Initial presentation with extensive colitis and high erythrocyte sedimentation rate (ESR) was associated with an increased risk of colectomy within 10 years. Patients diagnosed over the age of 50 years had a reduced risk for subsequent colectomy. Relapsing disease was noted in 83%, but half (48%) of the patients were relapse free during the last 5 years. 21% of patients with proctitis or left-sided colitis had progressed to extensive colitis within 10 years.(Solberg, Lygren et al. 2009).

Disease duration and extent of involvement of the colon increases the risk of colorectal cancer (CRC) as compared to sporadic CRC and the annual incidence rate of CRC in UC ranges from approximately 1 in 500 to 1 in 1600 (Loftus 2006).

1.2.4.2 Clinical assessment of Ulcerative Colitis

Clinical assessment of UC consists of evaluation of disease extent and disease activity. Distribution and activity of UC has been classified in the Montreal Classification according to the extent of the disease in proctitis (E1), left sided disease (E2) and extensive disease (E3) affecting the colon beyond the splenic flexure, including pan-colitis and the severity of symptoms such as remission (S0), mild (S1), moderate (S2) and severe disease (S3) (Silverberg, Satsangi et al. 2005). The Mayo score (Schroeder, Tremaine et al. 1987) provides a useful tool in evaluating patients with UC and has been used in this project. It focuses on typical symptoms of UC such as increased stool frequency, rectal bleeding and findings at endoscopic assessment and the conclusion of the physician regarding the current activity of the disease.

Table 4 provides an overview of the Mayo scoring system.

Mayo scoring system

Stool frequency

0 = Normal

1 = 1-2 stools/day more than normal

2 = 3-4 stools/day more than normal

3 = >4 stools/day more than normal

Rectal bleeding

0 = None

1 = Visible blood with stool less than half the time

2 = Visible blood with stool half of the time or more

3 = Passing blood alone

Mucosal appearance at endoscopy

0 = Normal or inactive disease

1 = Mild disease (erythema, decreased vascular pattern, mild friability

2 = Moderate disease (marked erythema, absent vascular pattern, friability, erosions)

3 = Severe disease (spontaneous bleeding, ulceration)

Physician rating of disease activity

0 = Normal

1 = Mild

2 = Moderate

3 = Severe

Table 4 Overview of the Mayo scoring system (Schroeder, Tremaine et al. 1987)

1.3 Drug therapy in IBD

Current drug treatments in IBD aim to induce and maintain the patient in remission and ameliorate the disease's secondary effects. Drug treatment of IBD generally uses the same set of medication for CD and UC; however, there are some differences that will be highlighted in this section. Generally medications used in the treatment of IBD are 5-ASA (AminoSAlicylates) preparations such as mesalazine (mainly in UC) (Camma, Giunta et al. 1997), topical and systemic steroids (Ford, Bernstein et al. 2011), the frequently used immune modulators such as the thiopurines (6-mercaptopurine and azathioprine) (Fraser, Orchard et al. 2002), and agents used on a less frequent basis such as methotrexate, tacrolimus and cyclosporine. Anti-TNF-α agents such as Infliximab and Adilumimab have given new treatment options in the last few years (D'Haens, Panaccione et al. 2011). Antibiotics have been used with limited success in specific scenarios especially in CD (Sandborn and Feagan 2003). In UC, post procto-colectomy and formation of an ileal pouch-anal anastomosis, antimicrobial therapy (ciprofloxacin, metronidazole) is an effective first-line treatment for acute pouchitis, and VSL#3, a probiotic bacterial formulation, has been shown to be superior to placebo for prevention of recurrence (Holubar, Cima et al. 2010).

Table 5 and ***Table 6*** give an overview of drugs used in UC and CD, respectively.

Drug	Induction	Maintenance	Distal UC	Pancolitis	Ileoanal Pouch
Biological therapy	No~	No~	No~	No~	Yes
Budesonide	No	No	Yes*	No*	Yes
Ciprofloxacin	No	No	No	No	Yes#
Immunomodulator	No	Yes	Yes	Yes	No
Mesalazine	Yes	Yes	Yes*#	Yes	No
Metronidazole	Yes	No	No	No	Yes*#
Prednisolone	Yes	No	Yes*#	Yes	No
VSL#3 (pouchitis)	No	Yes	No	No	Yes*

Table 5. Overview of drugs used in UC for induction of remission, maintenance and variations of disease extent and treatment of patients with ileoanal pouch. *topical, #oral, ~not in the UK

Drug	Induction	Maintenance	CD Ileocaecal	CD colitis	Fistulating CD
Budesonide	Yes	No	Yes	Possibly	No
Prednisolone	Yes	No	Yes	Yes	No
Immunomodulator	Yes	Yes	Yes	Yes	Yes
Biological therapy	Yes	Yes	Yes	Yes	Yes
Ciprofloxacin	No	No	No	No	Yes
Metronidazole	No	No	No	Yes	Yes

Table 6. Overview of drugs used in CD for induction of remission, maintenance and variations of disease extent and phenotype.

1.3.1 Aminosalicylates

1.3.1.1 Mechanism of Action

Multiple mechanisms of action have been demonstrated *in vitro* such as inhibition of the production of pro-inflammatory cytokines; e.g IL-1, TNF-α and induction of lymphocyte apoptosis (Doering, Begue et al. 2004), inhibition of the lipoxygenase pathway, scavenging of free radicals and oxidants contributing to cellular detoxification as well as inhibition of the inflammatory-related Nuclear Factor κB (NF-κB) pathway (Barnes and Karin 1997, Liptay, Bachem et al. 1999).

Another proposed mechanism of action of 5-ASA is via inhibition of IL-2 production in peripheral mononuclear cells inhibiting T-cell proliferation, changing cell adhesion expression pattern, reducing antibody production and mast cell release, and interfering with macrophage and neutrophil chemotaxis (Fujiwara, Mitsui et al. 1990).

PPAR-γ is a transcription factor modulating the inflammatory response of monocytes and macrophages by inhibiting the production of nitric oxide (iNOS) and cytokines such as TNF-α, IL-1 and IL-6 (Dubuquoy, Rousseaux et al. 2006). Expression is significantly reduced in inflamed mucosal UC tissue, which has been shown to be able to be restored by topical rosiglitazone, a PPAR-γ ligand (Pedersen and Brynskov 2010). Data on 5-ASA's has suggested a role for mesalamine as an additional ligand of PPAR-γ, which may explain some of its therapeutic effects in UC (Rousseaux, Lefebvre et al. 2005).

1.3.1.2 Clinical use in IBD

Aminosalicylates (5-ASA) remain the first-line therapy for both induction and maintenance of remission in mild-moderate UC. 5-ASA preparations were shown to be superior to placebo in maintaining endoscopic or clinical remission of UC and tended towards therapeutic benefit over sulfazalzine (Nguyen, Harris et al. 2006). Sulfazalzine has been used less in recent years due to its side effect profile. Rectal mesalazine as a suppository at 1g/day induces remission more effectively than placebo or topical steroids in distal UC (Cohen, Woseth et al. 2000, Marshall and

Irvine 2000), although both medications taken concomitantly are superior to mesalazine alone (Marshall and Irvine 2000).

5-ASA preparations have been reported to be chemo-protective against the development of colorectal cancer in patients with IBD. A meta-analysis of observational studies supports a protective association between 5-aminosalicylates and CRC/dysplasia in patients with ulcerative colitis (Velayos, Terdiman et al. 2005). The effect of 5-ASA preparations in the prevention of high grade dysplasia and colorectal cancer in patients with IBD appeared to be less pronounced in a large study from Van Schaik et al and the main benefit of chemoprevention was found to be with thiopurines (van Schaik, van Oijen et al. 2012).

The role of 5-ASA in induction of remission and maintenance therapy for CD is controversial, and there is no clear benefit of continued 5-ASA therapy in patients who achieve medical remission (Camma, Giunta et al. 1997).

1.3.2 Corticosteroids

1.3.2.1 Mechanism of Action

Glucocorticosteroids exhibit their function by binding to cytoplasmic glucocorticoid receptors found in most cell types resulting in a broad spectrum of effects on the immune system. Effects include inhibition of the recruitment and proliferation of lymphocytes, monocytes and macrophages, migration of neutrophils to sites of inflammation, and decreased production of soluble inflammatory mediators such as cytokines, leukotrienes, and prostaglandins (Katz 2004).

1.3.2.2 Clinical use in IBD

The European Cooperative Crohn's Disease Study (ECCDS) showed that 80% of patients with CD treated with methylprednisolone (48 mg/day) achieved remission at 18 weeks compared to less than 40% of placebo patients (Malchow, Ewe et al. 1984). Among patients with active Crohn's disease, both controlled-release budesonide and prednisolone are effective in inducing remission (53% and 66% at week 10 respectively). Budesonide has less systemic side effects than prednisolone and is used as a first line treatment for mild-moderate ileal CD (Rutgeerts, Lofberg et al. 1994).

In UC, for patients failing to achieve remission with 5-ASA preparations, corticosteroid agents in the form of oral prednisolone for mild to moderate UC, and intravenous steroid, such as hydrocortisone, for acute severe disease is recommended (TRUELOVE and WITTS 1955). In a study conducted by Kjeldsen J *et al.* treatment of acute flares of ulcerative colitis in 89 patients with doses of prednisolone above or equal to 40 mg resulted in an overall remission rate of 67%. Remission rate and colectomy rate were 47% and 42%, respectively, when the disease was severe, 80% and 13% when moderate, and 84% and 3% when mild to moderate (Kjeldsen 1993).

Use of corticosteroids has to be applied with caution due to its unfavourable side effect profile in 50% of patients. Early side effects caused by steroids include cosmetic, sleep and mood disturbance, dyspepsia, and glucose intolerance. Adverse effects from prolonged steroid use include cataracts, hypertension, hyperlipidaemia, osteoporosis, osteonecrosis, myopathy, and

susceptibility to infection (Katz 2004). Steroid withdrawal can lead to acute adrenal insufficiency, myalgia, malaise, and arthralgia or even raised intracranial pressure (Travis 2008).

1.3.3 Thiopurines

1.3.3.1 Mechanism of Action

6-Mercaptopurine and its pro-drug azathioprine (AZA) are purine analogues that are converted into 6-thioguanine nucleotides (TGN), which are the therapeutically active metabolites. 6-thioguanine nucleotides interfere with nucleic acid synthesis and exhibit anti-proliferative effects on activated lymphocytes (Tiede, Fritz et al. 2003, Schroll, Sarlette et al. 2005).

Intracellular activation of 6-MP through an initial step catalysed by the enzyme hypoxanthine phosphoribosyl transferase (HPRT) results in the formation of active thioguanine nucleotides (TGNs) (Lennard and Lilleyman 1996). The TGN metabolites act as purine antagonists and induce cytotoxicity and immunosuppression by inhibition of RNA, DNA, and protein synthesis. These cytotoxic properties are mainly due to the direct incorporation of TGN into DNA (Krynetskaia, Krynetski et al. 1999). The immunosuppressive effects of thiopurines could be mediated by binding of TGN triphosphate instead of guanine triphosphate to the Rac1 protein. This suppression of Rac1 activation leads to the induction of apoptosis (Tiede, Fritz et al. 2003). It has also been shown that thiopurines selectively inhibit inflammatory gene expression in activated T-lymphocytes (Thomas, Myhre et al. 2005).

Individual variations in drug metabolism are of importance for differences in tolerance to thiopurines partly explaining the varied side effect profile. Genetic polymorphisms in the TPMT gene are associated with decreased TPMT activity (Lindqvist, Haglund et al. 2004, Schaeffeler, Fischer et al. 2004) and with the development of myelotoxicity because of high TGN metabolite concentrations (Lennard, Van Loon et al. 1989).

1.3.3.2 Clinical use in IBD

For active CD disease, the overall response rate to thiopurines has been shown to be 54% for patients receiving treatment compared to 33% for those on placebo and for quiescent disease, an overall remission was seen in 67% of patients on treatment compared to 52% of those on placebo (Sandborn, Sutherland et al. 2000).

Azathioprine or 6-Mercaptopurine is usually started in a situation where patients have required more than one course of steroids to induce remission of CD or UC within the last calendar year. They are being used increasingly early in the disease course with the hope of reducing longer term end organ damage and improving long term patient outcomes.

In a study by Candy *et al.* sixty three patients with active CD were administered a three month taper of prednisolone while randomized to receive either AZA (2.5 mg/kg) or placebo. Although there was no difference in the number of patients achieving remission at week 12, 42% of the AZA group compared to 7% of the placebo group was in remission at 15 months (Candy, Wright et al. 1995).

In UC thiopurines have been shown to reduce steroid use and prevent relapse (Rosenberg, Wall et al. 1975, Kirk and Lennard-Jones 1982).

A large retrospective review of both CD (*n* = 272) and UC (*n* = 346) patients treated with AZA found a remission rate of 87% in those patients treated for more than six months compared to 59% overall (Fraser, Orchard et al. 2002).

Van Schaik FD et al studied a large population of IBD patients and found that Thiopurine use protects IBD patients against the development of high grade dysplasia and colorectal cancer. The effect of 5-ASA appeared to be less pronounced. (van Schaik, van Oijen et al. 2012)
Other immunosuppressant drugs administered in patients with IBD are methotrexate, tacrolimus, mycophenolate mofetil and ciclosporin (van Dieren, Kuipers et al. 2006). Ciclosporin has been used in rescue therapy for fulminant UC (Lichtiger, Present et al. 1994).

A double-blind controlled trial that randomized 20 patients with severe steroid-refractory UC to intravenous cyclosporin or placebo showed an 82% response rate in the cyclosporin group at a mean of seven days compared to zero in the placebo group, and over two-thirds of all responders avoided colectomy at six months (Lichtiger, Present et al. 1994).

In a recently published large multi-national multi-centre study Laharie et al showed that ciclosporin 2mg/kg and infliximab 5mg/kg are equally efficacious in achieving a clinical response of 85% after 7 days of treatment in patients with severe steroid refractory ulcerative colitis (Laharie, Bourreille et al. 2012).

1.3.4 Biologic agents

1.3.4.1 Mechanism of Action

Infliximab is a chimeric (75% mouse/25% human) anti-TNF-α monoclonal antibody whilst Adalimumab is a sub-cutaneously administered recombinant human IgG1 monoclonal antibody that binds with high specificity and affinity to human TNF-α (Hanauer, Feagan et al. 2002, Rutgeerts, Sandborn et al. 2005, Colombel, Sandborn et al. 2007, Reinisch, Sandborn et al. 2011).

Multiple mechanisms and sites of action have been described in in vitro and in vivo experiments.

Infliximab has been shown to neutralize soluble TNF-α (Van Den Brande, Peppelenbosch et al. 2002). In lamina propria T-cells, the induction of apoptosis by binding to trans-membrane TNF, induced caspase 3, caspase 8, Bax and Bak, which has been shown in in vitro experiments (ten Hove, van Montfrans et al. 2002, Van Den Brande, Peppelenbosch et al. 2002, Di Sabatino, Ciccocioppo et al. 2004, Mitoma, Horiuchi et al. 2005).

Ringheanu et al found that Infliximab induced “reverse signaling” in isolated PBMCs in vitro reducing the production of inflammatory cytokines. Monocytes produced less TNFα after they had been exposed to LPS when cultured with Infliximab. PBMC isolated from patients before and 1 h after INF infusion and stimulated with LPS produced less TNFα, IL-1β , IL-6, and IL-8

mRNA as measured by RT–PCR (Ringheanu, Daum et al. 2004). Activated T cells isolated from colonic biopsies of patients with CD cultured with Infliximab showed decreased expression of IFNγ (Agnholt and Kaltoft 2001).

Zeissig et al showed that patients with CD had a greater number of apoptotic epithelial cell foci than healthy controls. Patients who underwent treatment with Infliximab had epithelial barriers that were restored to normal (Zeissig, Bojarski et al. 2004).

1.3.4.2 Clinical use in IBD

The ACCENT I trial comparing patients with Crohn's disease who respond to an initial dose of Infliximab found that patients are more likely to be in remission at weeks 30 (placebo 21% versus Infliximab 5mg 39% versus Infliximab 10mg/kg 45%). The Infliximab groups were more likely to be in remission at week 54. Generally patients treated with Infliximab every 8 weeks were more likely to discontinue corticosteroids, and to maintain their response for a longer period of time (Hanauer, Feagan et al. 2002).

In the ACCENT II study, which investigated the effect of Infliximab on draining abdominal or perianal fistulas in CD, found that at week 54, 36% in the Infliximab group compared to 19% in the placebo group had no draining fistulas (Sands, Anderson et al. 2004).

In UC ACT 1 (refractory or intolerant to steroids and/or azathioprine/6-mercaptopurine) and ACT 2 (refractory or intolerant to 5-ASA agents) studies demonstrated a beneficial effect of Infliximab and showed that approximately two-thirds in the Infliximab group achieved clinical response and one-third achieved long-term remission, while 22% discontinued steroids (Rutgeerts, Sandborn et al. 2005).

For acute fulminant ulcerative colitis Infliximab was inferior to ciclosporin as a rescue therapy given that colectomy frequencies were significantly higher with biologic therapy both at 3 and 12 months' follow-up (Sjoberg, Walch et al. 2011).

In a recently published large multi-national multi-centre study Laharie et al showed that ciclosporin 2mg/kg and infliximab 5mg/kg at weeks 0, 2 and 6 are equally efficacious in achieving a clinical response of 85% after 7 days of treatment in patients with severe steroid

refractory ulcerative colitis. After 98 days, treatment failure (absence of response at day 7, relapse between day 7 and 98, absence of steroid-free remission at day 98, severe adverse events resulting in treatment interruption, colectomy, or death) occurred in 35 (60%) of 58 patients receiving ciclosporin compared with 31 (54%) of 57 receiving infliximab (Laharie, Bourreille et al. 2012).

The CHARM trial (n = 854) examined Adalimumab induction and maintenance efficacy in patients with moderately to severely active CD and 499 patients (58%) achieved clinical response after induction. Thereafter patients were randomized to Adalimumab 40 mg every other week, or 40 mg weekly or placebo, through to week 56. Significantly higher rates of remission were seen in the Adalimumab groups compared to placebo at both week 26 (40% and 47% versus 17%) and week 56 (36% and 41% versus 12%). The Adalimumab groups also had significantly more steroid discontinuation and complete fistula closure (Colombel, Sandborn et al. 2007).

A recent study investigating the efficacy of Adalimumab in moderate to severe UC showed an 18.5% remission rate at week 8 as compared to 9.2% in the placebo group (Reinisch, Sandborn et al. 2011).

1.3.5 Medical management of IBD

1.3.5.1 Medical management of CD

Treatment of Crohn's disease aims to achieve lasting clinical and endoscopic remission (mucosal healing) and to interrupt the naturally progressive destructive disease course that can lead to intestinal failure and associated complications (Baumgart and Sandborn 2012).

First-line therapy for mild Crohn's disease is dependent on the disease location and phenotype of the disease. Patients with mild ileal or ileo-caecal disease are usually induced into remission with budesonide at 9 mg daily (Baumgart and Sandborn 2007).

In moderate to severe ileal, colonic or ileo-colonic Crohn's disease a fast acting short-term use agent (ie, steroids or anti-TNF) to achieve rapid symptom relief and disease control is combined with thiopurines or methotrexate for long-term maintenance (Baumgart and Sandborn 2012). Induction therapy with 40mg oral prednisolone per day in a reducing dose or biologic agents such as Infliximab at 5 mg/kg bodyweight at weeks 0, 2, and 6 or Adalimumab given subcutaneously as a loading dose of 160 mg at week 0 and 80 mg at week 2 and 40mg at week 8 can be used while starting azathioprine or 6-mercaptopurine for maintenance of remission which take up to 17 weeks to be fully effective. If a biological agent is being used as an induction agent increasingly it is continued as a maintenance treatment often in combination with immunomodulators.

The main maintenance of remission agents for CD are azathioprine 2.5 mg/kg bodyweight or 6-mercaptopurine 1.5 mg/kg bodyweight. An alternative to azathioprine or 6-mercaptopurine is maintenance therapy with methotrexate at 15 to 25 mg per week given intramuscularly or subcutaneously specifically useful in patients with enteropathic arthritis. 5-ASA agents are not effective for maintenance of remission in patients with CD. Budesonide maintenance of therapy modestly prolongs the time to relapse (Baumgart and Sandborn 2007). Maintenance therapy of 5 mg/kg bodyweight of Infliximab every 8 weeks, or Adalimumab given subcutaneously at 40 mg every other week should be considered alongside immunosuppressive therapy with azathioprine, 6-mercaptopurine, or methotrexate to achieve a steroid-free remission in patients who relapse whilst on immunomodulators. Combination therapies with thiopurines and TNF

blockers have been shown to be superior for improved symptom control and mucosal healing compared to mono-therapy in newly diagnosed patients with Crohn's disease. (D'Haens, Baert et al. 2008). Surgery should be contemplated in patients with obstructive complications (strictures) and those who have not responded to medical therapy (Baumgart and Sandborn 2007).

In patients with fulminant inflammatory CD, remission might be induced with intravenous corticosteroids such as hydrocortisone 100mg 3-4 times a day. Intravenous Infliximab at a dose of 5 mg/kg bodyweight at weeks 0, 2, and 6 is an alternative main therapy in these patients and can be used in patients who do not respond to intravenous corticosteroids. An alternative to Infliximab is Adalimumab given subcutaneously as a loading dose of 160 mg at week 0 and 80 mg at week 2 and 40mg every other week thereafter. Surgery might be an appropriate initial therapy for patients with fulminant ileo-caecal disease with obstructive complication or those unable to tolerate medical therapy (Baumgart and Sandborn 2007).

Fistulising CD requires close liaison between surgeons and gastroenterologists. Remission might be induced with antibiotics (ciprofloxacin at 1000mg per day or metronidazole at 1000–1500mg per day), immunomodulators such as azathioprine or methotrexate or Infliximab at a dose of 5 mg/kg bodyweight at weeks 0, 2, and 6 should be considered once all sepsis has been successfully drained with either a fistulotomy or drainage with setons or both. An alternative to Infliximab is Adalimumab, which is given subcutaneously as 160 mg at week 0 and 80 mg at week 2, and then 40 mg subcutaneously every other week beginning at week 4. Patients with fistulising CD can be maintained on azathioprine 2.5 mg/kg bodyweight or 6-mercaptopurine at 1.5 mg/kg bodyweight monotherapy or combined with Infliximab 5 mg/kg bodyweight every 8 weeks as maintenance therapy (Mowat, Cole et al. 2011).

1.3.5.2 Medical management of UC

1.3.5.2.1 Induction of Remission

5-ASA compounds (oral and/or topical) are used to achieve induction of remission in mild to moderately active UC. If topical therapy is used, suppositories are most appropriate for proctitis, whereas more extended disease affecting the sigmoid or greater parts of the left colon need the addition of enemas or foams. Oral (2.4g-4.8g) or topical (1g) 5-ASA doses are sufficient to induce remission. Oral or topical 5-ASA should be continued as maintenance therapy in patients who respond to induction therapy with 5-ASA (Mowat, Cole et al. 2011).

Moderately active UC that does not respond to 5-ASA or severe (but not fulminant) disease usually requires treatment with oral corticosteroids at a dose of 40mg of prednisolone per day in a reducing dose regime with concomitant 5-ASA treatment. Patients needing steroid therapy more than once a year should be commenced on disease modifying drugs such as the thiopurines, azathioprine at 2.5 mg/kg bodyweight per day or 6-mercaptopurine at 1.5 mg/kg bodyweight per day (Mowat, Cole et al. 2011).

Biologic agents such as Infliximab at a dose of 5 mg/kg bodyweight at 0, 2, and 6 weeks followed by maintenance therapy every 8 weeks or Adalimumab given subcutaneously as a loading dose of 160 mg at week 0 and 80 mg at week 2, followed by a maintenance dose of 40 mg every 2 weeks could be considered in outpatients with steroid dependent or refractory disease, but maintenance therapy with biologic agents is currently not recommended in the UK (Mowat, Cole et al. 2011).

Fulminant colitis requires close collaboration of gastroenterologists and surgeons to ensure appropriate referral for emergency colectomy if medical treatment is unsuccessful. Medical therapy is indicated in patients with severe UC who do not seem toxic (abdominal tenderness or sepsis). Intravenous corticosteroids, such as Hydrocortisone 100mg 4 times per day, are the first line of treatment with supportive therapy such as intravenous fluids. Bowel rest or parenteral nutrition are necessary in patients with severe UC but should be strongly considered in patients with toxic megacolon, where surgery might be imminent. Broad-spectrum antibiotics are not indicated in the absence of abdominal infection. Rescue medical therapy or surgery should be

considered in patients who do not improve significantly after 3-5 days of intravenous corticosteroids. Ciclosporin, Tacrolimus, and Infliximab are all effective as rescue therapies. Ciclosporin is given intravenously as a 24-h continuous infusion at doses of 2mg/kg bodyweight per day. Ciclosporin 2mg/kg and infliximab 5mg/kg at weeks 0, 2 and 6 are equally efficacious in achieving a clinical response of 85% after 7 days of treatment in patients with severe steroid refractory ulcerative colitis, but infliximab may have slightly more favourable side effect profile (Laharie, Bourreille et al. 2012). Tacrolimus is dosed orally to achieve a serum concentration of 5–15 ng/mL. No response to medical rescue therapy is an indication for colectomy (Mowat, Cole et al. 2011).

1.3.5.2.2 Maintenance of Remission

The mainstay of maintenance of remission in ulcerative colitis is continuous treatment with 5-ASA preparations. Most patients can stay in remission using oral once-daily mesalazine at doses of 1·6–3·0 g per day, maintaining remission rates of about 70–90% (Kruis, Jonaitis et al. 2011).

Patients who relapse despite optimum doses of mesalazine, those with steroid dependency, and those previously treated with cyclosporine or tacrolimus for a severe flare, should be given thiopurines such as azathioprine at 2.5 mg/kg bodyweight per day or 6-mercaptopurine at 1.5 mg/kg bodyweight per day (Gisbert, Linares et al. 2009).

Maintenance therapy with biologic agents in ulcerative colitis is currently not recommended in the UK (Mowat, Cole et al. 2011).

1.3.6 Endoscopy in IBD

Endoscopy in IBD serves the purpose of assessing the severity and extent of the disease as well as its activity and there is a therapeutic role for endoscopy, particular in the removal of polyps and dysplastic lesions as well as treatment of strictures. Establishing the phenotype of disease in IBD as per the Montreal Classification is important for planning and choice of treatment. Assessment of mucosal healing by endoscopy in IBD following therapy is an important indicator of treatment success. Colonoscopy is also required for surveillance of patients with IBD due to the raised risk of colorectal cancer in IBD as compared to the normal population. According to Gillen *et al*. there is an 18-fold increase in the risk of developing colorectal cancer in extensive Crohn's colitis and a 19-fold risk increase in extensive ulcerative colitis when compared with the general population (Gillen, Walmsley et al. 1994).

Endoscopists should obtain both targeted (lesion-directed) and non-targeted biopsies of the colon and rectum. Current recommendations suggest that all patients should undergo endoscopic evaluation 8-10 years after diagnosis of ulcerative colitis or Crohn's colitis to reassess disease extent and severity in order to plan subsequent endoscopic surveillance strategy. Patients are risk stratified at subsequent colonoscopies according to extent of disease, grade of inflammation and independent risk factors of colorectal cancer. Surveillance is recommended in the low risk group every 5 years. For patients with medium risk 3-yearly colonoscopy is indicated. Yearly colonoscopy is reserved for high risk patients with extensive colitis (either ulcerative colitis or Crohn's colitis) with moderate or severe endoscopic/histological active inflammation or stricture within past 5 years. Patients with IBD with confirmed dysplasia within past 5 years, not opting for colectomy, and patients with Primary Sclerosing Cholangitis (PSC) are an exception to the usual surveillance guidelines because of their heightened risk of CRC. In PSC patients surveillance is recommended at diagnosis and yearly after a diagnosis of PSC is established. Comparing the cumulative risk of colorectal neoplasia (cancer or dysplasia) after 25 years of disease duration in patients with IBD and PSC to IBD without PSC the risk was 50% and 10% respectively (Broome, Lofberg et al. 1995). Primary sclerosing cholangitis/post-orthotopic liver transplant for primary sclerosing cholangitis or a family history of colorectal cancer in a first-degree relative aged <50 years are also surveyed on a yearly basis (Cairns, Scholefield et al. 2010).

To exclude dysplasia in colonic mucosal biopsies with 95% confidence at least 56 non-targeted jumbo-forceps biopsies need to be obtained at each endoscopic surveillance examination. 90% confidence was achieved with 33 biopsies (Rubin, Haggitt et al. 1992). The use of chromoendoscopy has been implemented by many centres for colitis-related surveillance and is recommended by the British Society of Gastroenterology (Cairns, Scholefield et al. 2010).

Targeted biopsies with chromoendoscopy improve the sensitivity and specificity for the detection of discret colonic lesions. Crypt architecture can be categorized by evaluating the pit pattern helping the endoscopist in differentiation of neoplastic from non-neoplastic changes. Several investigators have documented an increased yield of dysplasia detection using either diluted methylene blue or indigo carmine spray compared to conventional colonoscopy (Kiesslich, Fritsch et al. 2003, Rutter, Saunders et al. 2004, Hurlstone, Sanders et al. 2005). Newer endoscopic techniques include narrow-band imaging (Dekker, van den Broek et al. 2007) and endomicroscopy.

An essential issue in IBD surveillance is the management of adenoma-like DALMs (Dysplasia-Associated Lesion or Mass) and whether a dysplastic lesion can be safely completely removed endoscopically. Raised lesions are commonly encountered during surveillance colonoscopy (Friedman, Odze et al. 2003). A retrospective study of 56 patients with dysplasia showed that 50 patients (89.3%) had macroscopically detectable neoplastic lesions. (Rutter, Saunders et al. 2004). In a similar study from the United States, Rubin and colleagues demonstrated that 38 of 65 dysplastic lesions (58.5%) and 8 of 10 cancers (80.0%) were visible endoscopically (Rubin, Rothe et al. 2007). The management of adenoma-like DALMs depends on the size and appearance of the lesion and the ability of the endoscopist to detect and entirely remove such lesions at colonoscopy, otherwise colectomy is an alternative.

Endoscopic dilatation is an effective and safe treatment for short strictures caused by Crohn's disease. Meta-analysis of thirteen studies accounting to a total of 347 Crohn's disease patients found that endoscopic dilatation was mainly applied to postsurgical strictures, being technically successful in 86% of the cases. Long-term clinical improvement was achieved in 58% of the patients with a follow up of up to 33 months. Major complication rate was 2%, being higher than 10% in two series. At multivariate analysis, a stricture length of less or equal to 4 cm was associated with a surgery-free outcome (Hassan, Zullo et al. 2007).

1.4 Mucosal barrier and innate and adaptive immunity in IBD

IBD represents a complex series of interactions between susceptibility genes, the environment, the mucosal barrier and the immune system. Genes coding for the mucosal barrier, bacterial sensing and the innate and adaptive immune system have been identified as candidates in GWAS (see section 2.1.6.1). In addition to this, the effect of immunosuppressive drugs on the clinical features of the disease and histo-pathological findings suggest a significant involvement of the immune system in the pathogenesis of the disease. Various aspects of the mucosal immune system are involved in the pathogenesis of IBD including luminal antigens, intestinal epithelial cells, cells of the innate and adaptive immune system, and their secreted mediators.

1.4.1 Intestinal mucosal barrier

The main function of the intestinal epithelium consists of the absorption of water and nutrients but also has a vital role in acting as a barrier to luminal pathogens. The mucosal barrier in the intestinal tract is a monolayer of columnar intestinal epithelial cells including specialist cells such as Paneth cells and mucus secreting goblet cells, as well as auto-endocrine entero-chromaffine cells. The epithelial monolayer is carefully folded, producing crypts and villous protrusions. Individual epithelial cells are intimately connected to each other through tight junctions and serve as a physical protective layer. The intestinal epithelium separates luminal contents from the underlying immune compartments and provides an efficient barrier to block the entry of microflora into the lamina propria (Snoeck, Goddeeris et al. 2005, Simon-Assmann, Turck et al. 2007, Ramasundara, Leach et al. 2009).

The three main junctions between these epithelial cells are tight junctions, adherens junctions and desmosomes. Tight junctions form a continuous, circumferential barrier towards the luminal side of the intercellular junction and consist of three main trans-membrane proteins: occludin, claudins, and junctional adhesion molecules (Hossain and Hirata 2008). Tight junctions prevent influx of pathogens and antigens and help to maintain the barrier between the sub-mucosa and the luminal contents. Pro-inflammatory cytokines such as IFN-γ and TNF-α as well as the T helper type 2 (Th2) pro-inflammatory cytokine IL-13 cause an impairment of tight

junctions by down regulating junctional proteins (Prasad, Mingrino et al. 2005, Lu, Huang et al. 2011). In contrast, TGF-β produced by regulatory T cells can preserve the integrity of the junctions (Mankertz, Tavalali et al. 2000, Bruewer, Luegering et al. 2003, Ye, Ma et al. 2006, Heller, Fromm et al. 2008).

Specialized intestinal epithelial cells in the form of mucus-secreting goblet cells and antimicrobial peptide producing Paneth cells form an indiscriminate layer of protection as part of the mucosal barrier against luminal bacterial antigens (Artis 2008). Goblet cells are the main contributors in the production of the mucus layer coating the gastrointestinal tract and play an important role in host protective immunity. The development of intestinal goblet cell hyperplasia/hypertrophy in an inflammatory response involves the Th2 cytokines IL-4 and IL-13 via STAT6 activation. These specialised epithelial cells synthesize secretory and membrane bound mucin glycoproteins forming a viscous layer of defence (Kim and Ho 2010). Blanchard *et al* demonstrated that IL-4 and IL-13 up-regulate the release of specific goblet cell product trefoil factor-3 (TFF3) from HT-29 cells, a mucus-producing colonic epithelial cell line. This was observed in a time- and dose-dependent fashion under a Th2-mediated inflammatory scenario via STAT6 activation and was accompanied by up-regulation of the goblet cell product mucin 2 (MUC2) (Blanchard, Durual et al. 2004).

Defensins are antimicrobial peptides that are secreted into the mucous layer. They are produced by Paneth cells (α- defensins) in the small bowel and by plasma cells and epithelial cells in the large bowel (β- defensins). Defensins contribute to host immunity and assist in maintaining the balance between protection from pathogens and tolerance to normal flora (Ramasundara, Leach et al. 2009).

Secretory IgA (Immunoglobulin A) is produced by lamina propria plasma cells (Wershil and Furuta 2008). It also contributes to the mucosal barrier by preventing luminal antigens, microorganisms, and other foreign proteins from breaching the epithelial monolayer, and can neutralize toxins and infectious organisms (Mestecky, Russell et al. 1999).

Genes associated with epithelial barrier function have also been identified as being implicated in UC susceptibility in GWAS such as ECM1, CDH1, HNF4a, and LAMB1 regulating mucosal permeability (Thompson and Lees 2010).

1.4.2 Innate and adaptive intestinal mucosal immunity and the intestinal flora in IBD

The intestinal mucosa is host to a vast number of resident bacteria of the gut flora and other nutrients and pathogens passing through the digestive tract. Pattern Recognition Receptors (PRRs) expressed on cell surfaces (Toll-like receptors) or in the cytoplasm (NOD-like receptor proteins) are constantly monitoring the luminal content for potentially hazardous material. PRRs include different types of receptors such as the family of Toll-like receptors (TLRs), C-type lectins, Nucleotide-binding domain and Leucine-rich repeat-containing Receptors (NLRs) and retinoic acid-inducible gene I-like receptors. These various receptors survey different parts of the cells such as the epithelial cell surface as well as lysosomal and cytoplasmatic locations, recognizing conserved structures of microorganisms called pathogen-associated molecular patterns (PAMPs) and activating the mucosal immune system (Rakoff-Nahoum and Medzhitov 2008).

Evidence of the importance of the microbial gut flora in IBD has also been provided by animal studies. In murine transgenic models where colitis did not develop in a sterile germ-free environment, intestinal inflammation rapidly emerged when mice were reconstituted with luminal flora bacteria (Hata, Andoh et al. 2001, Rath, Schultz et al. 2001).

Sampling of gut luminal antigens activates the innate mucosal immune response consisting of dendritic cells (DCs), Paneth cells, macrophages and neutrophils. This immune activation is mainly mediated by enhanced Toll-like receptor (TLR) activity and it determines whether an antigen is tolerated or whether action needs to be taken to eliminate it. If an antigen is recognised as foreign and not tolerated, the stimulated DCs initiate the differentiation of naïve T-cells into effector T-cells regulated by IL-10 and TGF-β. Depending on the antigen and the cytokine milieu, effector T-cells then either develop one of three adaptive immune responses: Th1, Th2 or Th17. CD is a mainly Th1 and Th17 mediated process, whereas UC seems to be predominately mediated through Th2 and NK T-cells producing IL-13 (Shih and Targan 2008, Matricon, Barnich et al. 2010, MacDonald, Monteleone et al. 2011, Strober and Fuss 2011).

In the healthy gut, lamina propria macrophages are sampling and eliminating bacterial antigens that have breached the mucosal barrier. This is achieved without causing any significant pro-inflammatory signals through production of the immune-regulatory cytokine IL-10 (Denning,

Wang et al. 2007, Bar-On, Zigmond et al. 2011). During inflammation, lamina propria intestinal macrophages display pro-inflammatory profiles including the production of IL-12, IL-23, inducible NO Synthase (iNOS) and TNFα (Takada, Ray et al. 2010, Bar-On, Zigmond et al. 2011).

Polymorphmonuclear neutrophils transmigration across mucosal epithelia is a histological hallmark of inflammatory conditions such as UC and CD (Chin and Parkos 2006). Infiltration of the mucosa and sub-mucosa with neutrophils occurs in response to epithelial cells secretion of chemotactic signals such as interleukin-8 (IL-8), a potent neutrophil chemoattractant produced in response to pathological stimuli (Kucharzik, Hudson et al. 2005). Neutrophil infiltration is also facilitated by IL-1β and TNF-α that increase the production of selectins and integrins and weaken the intercellular junctions (Alzoghaibi 2005). Neutrophils which are trans-located to the mucosa and sub-mucosa produce pro-inflammatory substances such as reactive oxygen species (ROS), TNFα, IL-1 and proteases causing tissue inflammation (Weiss and LoBuglio 1982, Rogler and Andus 1998).

The mucosal immune system of the gastrointestinal tract can react through the Gut-Associated Lymphoid Tissue (GALT) system directed by antigen presenting dendritic cells in specialised areas called Peyer's patches. Peyer's patches are lymphoid aggregates made up of a large B-cell follicle, an interfollicular T-cell region, and numerous intervening macrophages and DCs (Wershil and Furuta 2008). It is important for the gut to be able to distinguish between pathogens and to tolerate commensal bacteria and antigens related to nutrition, known as oral tolerance. Disruption of mucosal immune homeostasis in genetically susceptible individuals results in altered handling of enteric antigens leading to pathogenic T cell activation and chronic inflammation (Ott, Musfeldt et al. 2004, Backhed, Ley et al. 2005, Manichanh, Rigottier-Gois et al. 2006).

The overlying epithelial cells are called M cells (human micro-fold cells). These cells are specialist epithelial cells that differ from absorptive epithelium by the absence of microvilli and membrane-associated hydrolytic enzymes as well as the presence of a reduced glycocalyx. M cells can release pro-inflammatory cytokines and chemokines. In addition, they are also capable of transporting antigens and pathogens directly to the sub-epithelial lymphoid tissue through trans-epithelial vesicular transport (Miller, Zhang et al. 2007).

Antigen presentation is facilitated by dendritic cells, macrophages or directly through M cells in the Peyer's plaques or sub-mucosa to naïve T-cells and regulated by IL-10 and TGF-β. Antigen presentation influences the balance of regulatory and effector T-cells, predominating effector T cells over regulatory T cells. Mucosal suscesptibility or defective sampling of antigens could lead to activation of the submucosal innate immune system tipping the pro- and anti-inflamatory balance towards inflammation (Shih and Targan 2008). Mucosal T cells are important in the induction and persistence of chronic inflammation by producing pro-inflammatory cytokines. Th1-related cytokines such as TNF-α, IFN-γ or IL-12 as well as Th17-associated cytokines (e.g. IL-17A, IL-21, IL-23) are up-regulated in inflamed mucosa of CD. In contrast, cytokine profiling in inflamed UC shows increased production of Th2-related cytokines such as IL-5 and IL-13, the latter produced by natural killer T cells (NKT) (Ott, Musfeldt et al. 2004, Backhed, Ley et al. 2005, Manichanh, Rigottier-Gois et al. 2006, Strober and Fuss 2011).

1.5 The role of IL-13 in the pathogenesis of UC

UC is driven by a Th2-like response whose major factor is believed to be NKT cells producing IL-13 (and IL-5) in humans (Strober and Fuss 2011). The first evidence emerged through murine animal studies using dermal oxazoline pre-sensitization before rectal administration of oxazoline. This led to a more prolonged distal colitis that was defined by an initial IL-4 response superseded after approximately 4–5 days with an increasing IL-13 response. It was established that NKT cells as well as IL-13 have a negative effect on epithelial cells leading to increased mucosal permeability through apoptosis and alteration of tight junctions (Heller, Fuss et al. 2002). Futher murine models of UC were studied by Rosen et al which used STAT6 deficient mice which will be discussed later in this paragraph (Rosen, Chaturvedi et al. 2013). Generally murine models have been helpful in IBD research but findings could not always be confirmed in humans.

Futher work from Heller *et al.* using electrical resistance measurements in epithelial cells showing that IL-13 exposure decreases the mucosal electrical resistance in HT-29 epithelial cells and also increases the production of claudin-2, leading to increased epithelial barrier permeability in UC (Heller, Florian et al. 2005).

Human studies looking at lamina propria mononuclear cells have shown marked differences on cells bearing the NKT cell marker (CD161) between normal colon, CD and UC. Mononuclear cells from UC specimens produced IL-13; in contrast, those from CD disease specimens showed production of IFN-γ. Interestingly, the production of IL-13 by NKT cells could only be achieved by antigen-presenting cells bearing CD1d, a surface glycogen, which facilitates a non-classical CD1-dependent NKT cell activation; in contrast, CD cytokines were produced from classically activated Th1 CD4+ T cell (Fuss, Heller et al. 2004).

IL-13, up-regulated in UC, alongside IL4 induces activation of the JAK–signal transducer and activator of transcription 6 (STAT6) via IL-13 Receptor alpha 1 (IL13Rα1) (Fuss, Heller et al. 2004).

IL-13 has a higher affinity to the IL-13Rα2 receptor which appears to function largely as a scavenger for IL-13. By binding IL-13 and making it unavailable for activating the IL13Rα1, while not inducing its own intracellular signal, it acts as a decoy receptor regulating the IL-13 pathway

activation (Kasaian, Raible et al. 2011). IL13Rα2 is also thought to exhibit its own signalling exhibiting an inhibitory function by down regulating STAT6 and increasing the production of TGF-β as shown in a murine model (Fichtner-Feigl, Young et al. 2008).

In bronchial epithelial cells Yasunaga *et al* have shown that co-expression of the IL-13Rα1 and IL-13Rα2 leads to inhibition of the IL-13 induced phospho-STAT6 production. The mechanism of this inhibition is attributed to the preferential binding of IL-13 to IL-13Rα2 since this inhibition can be overcome with excess IL-13 (Yasunaga, Yuyama et al. 2003), similar to the deregulation of this balance in active ulcerative colitis.

STAT-mediated signal transduction plays a pivotal role in IBD inflammatory processes, which are characterized by a mucosal cytokine imbalance (Bouma and Strober 2003, Mudter, Weigmann et al. 2005).

Rosen, M. J *et al* found that UC is associated with increased colonic epithelial STAT6 phosphorylation. STAT6 inhibition with siRNA prevented IL-13-induced apoptosis and barrier disruption. Moreover, the HDAC-inhibitor SAHA inhibited IL-13-induced STAT6 phosphorylation and claudin-2 expression. In addition, SAHA also mitigated IL-13-induced reduction in trans-epithelial resistance in HT-29 cells. Together, these findings make STAT6 a possible therapeutic target in UC (Rosen, Frey et al. 2011).

Rosen et al also showed that by inducing colitis in a murine model of UC, in STAT6-deficient (STAT6(-/-)) as compared to wild type (WT) mice, a decreased epithelial cell, T cell, macrophage, and NKT cell STAT6 phosphorylation was observed. Colitis was attenuated in STAT6(-/-) mice, with improvements in weight, colon length, and histopathology. There was decreased induction of the pore-forming tight junction protein claudin-2 in STAT6(-/-) mice. Mesenteric lymph node cells from STAT6(-/-) mice with colitis exhibited reduced secretion of IL-4, IL-5, IL-13, and IFN-γ. These data implicate STAT6 in the pathogenesis of colitis in vivo with important roles in altering epithelial barrier function and regulating Th2-inducing cytokine production (Rosen, Chaturvedi et al. 2013)

Therefore, a balance in the immune response is required for a healthy intestinal mucosa and involves both activation and repression signals. IL-4 and IL-13- dependent activation of STAT6

through IL13Rα1 is inhibited by Suppressor of Cytokine Signalling 1 (SOCS1) (Dickensheets, Venkataraman et al. 1999). Type 1 interferons have been shown to influence STAT6 expression via SOCS1 by down-regulation of IL-13 and IL-4 production in macrophages (Dickensheets, Venkataraman et al. 1999). A clinical study by Mannon P. J *et al* was able to show that Interferon-β-1a, a type I interferon, induces clinical response and remission in a large subset of patients with ulcerative colitis. This was associated with significant inhibition of IL-13 production and seemed to be facilitated via SOCS proteins (Mannon, Hornung et al. 2011). Although SOCS1 is an inhibitor of STAT6 activation through IL-13 via IL13Rα1, its activation is early and may be an immediate negative feedback loop for excessive STAT6 activation (Hebenstreit, Luft et al. 2003).

Other factors that seem to play a part in the Th2 like inflammatory response in ulcerative colitis in connection with IL-13, are IL-25, Thymic stromal lymphopoietin (TSLP) and the Matrix metalloproteinases (MMPs) MMP9 and MMP12.

IL-25, known to enhance IL-13 production (Fort, Cheung et al. 2001), has been demonstrated to be a pro-inflammatory cytokine in a type-2 model of murine dextran sulfate sodium (DSS) colitis. Neutralizing IL-25 prior to the onset of colitis almost completely reversed the physical symptoms of disease (Mchenga, Wang et al. 2008).

TSLP is an inflammatory Th2 regulator that is increased in the mucosal lesions of UC and increased TSLP expression by IECs may trigger exacerbation of UC (Tanaka, Saga et al. 2010).

MMP-9 is up-regulated in IBD (Garg, Vijay-Kumar et al. 2009) and MMP-12 was shown to be abundantly expressed by plump-macrophage-like cells of the inflamed lamina propria in ulcerative colitis (Vaalamo, Karjalainen-Lindsberg et al. 1998).
MMP9 and MMP12 have both been implicated in Th2 driven IL-13 dependent inflammation and fibrosis in mouse models of liver and lung (Madala, Pesce et al. 2010).

1.6 microRNAs

MicroRNAs (miRs) were first described by Ambros *et al* (lin-4 from *Caenorhabditis elegans*) in 1993 as an endogenous regulator of developmental timing genes (Bartel 2004). Together with short-interfering RNAs (siRNAs) and Piwi-RNAs (piRNAs), miRs belong to the RNA interference (RNAi) pathway. MicroRNAs act mainly as a negative regulators of gene expression through direct targeting of mRNAs (Carthew and Sontheimer 2009).

MicroRNAs are small non-coding RNAs of approximately 18-25 nucleotides in length that inhibit gene expression by pairing to the 3' UnTranslated Region (3'UTR) of their target mRNAs. This interaction facilitates degradation or translational repression of the mRNAs (Bartel 2004).

MicroRNAs are transcribed as a double-stranded primary transcript (pri-miR) by RNA polymerase II; pri-miRs get then processed by the nuclear enzyme Drosha (a ribonuclease III) into a double-stranded microRNA precursors of ~70 nulcleotides (pre-miR) which is trans-located into the cytoplasm via Exportin 5 (Bartel 2004, Boyd 2008, O'Connell, Rao et al. 2010, Ha 2011). Pre-miRs are then processed by Dicer, an enzyme that cleaves these precursors into the 22-nucleotide double-stranded mature microRNAs. This duplex is then unravelled and the leading strand ("guide strand") is incorporated into the RNA-Induced Silencing Complex (RISC) while the passenger strand is degraded (Boyd 2008, O'Connell, Rao et al. 2010). MicroRNAs integrated in the RISC are able to bind to the 3' UnTranslated Region (3' UTR) of target mRNAs blocking translation or causing mRNA degradation (Bartel 2004). However, miRNAs might enhance translation in some circumstances. For example, in resting macrophages, the 3'-UTR of TNF-α mRNA has been shown to enhance its expression upon lipopolysaccharide (LPS) stimulation with co-administration of miR-155 (Tili, Michaille et al. 2007).

MicroRNAs are thighly regulated through multiple processes on transcriptional (epigenetic, transcription factors), microRNA processing (Drosha, Expotin 5, Dicer) and transcriptional (RISC complex, AGO proteins) level (Davis and Hata 2009).

Hundreds of microRNAs encoded in the human genome and thousands of target mRNAs have been identified highlighting the crucial gene regulatory function of microRNAs. MicroRNAs have

been shown to be key in biological processes such as development, differentiation and proliferation, as well as in inflammation and apoptosis pathways (Ha 2011). In humans, microRNAs are thought to regulate about 30% of the protein-coding genes and individual microRNAs typically target potentially a large number of transcripts rather just one specific gene (Rajewsky and Socci 2004).

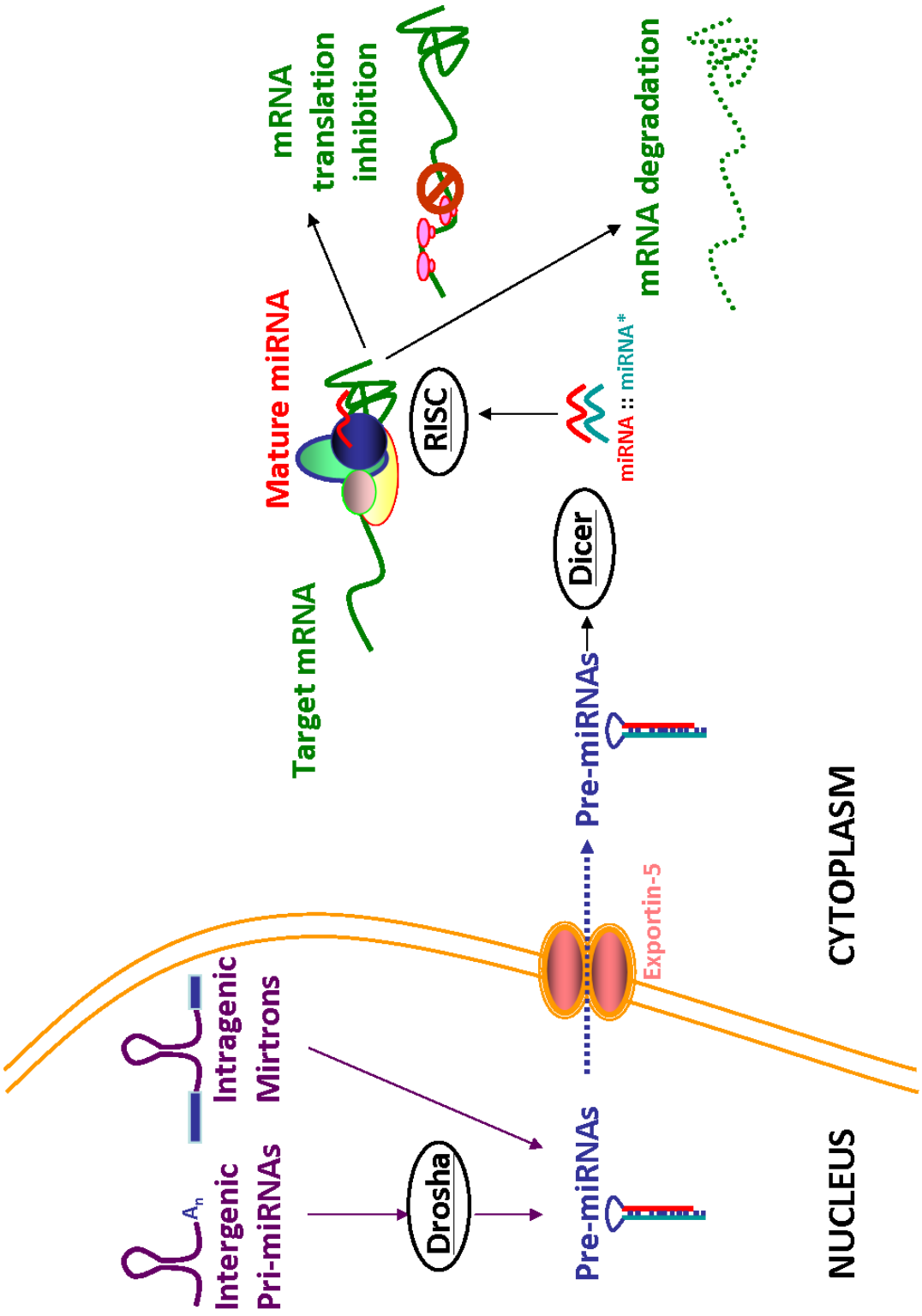


Figure 2. Biogenesis of microRNAs. Starting from gene transcription, pri-microRNAs are processed to pre-microRNsA by DROSHA; pre-microRNAs are translocated via EXPORTIN 5 to the cytoplasm, where they are cleaved by DICER. The guide strand is loaded into the RISC complex where it binds to its target mRNA leading to translational regression or degradation.Adapted from Martinez-Nunez R.T. (PhD Thesis March 2011).

1.6.1 Examples of the role of microRNAs in innate and adaptive immunity

More than 100 microRNAs are expressed by the cells of the innate and adaptive immune systems and have a profound impact on immune-regulatory processes (O'Connell, Rao et al. 2010). This is evidenced by the dysregulation of microRNAs in many inflammatory and immunological diseases (Sonkoly and Pivarcsi 2009, Furer, Greenberg et al. 2010, Iborra, Bernuzzi et al. 2010) as well as their essential function in immune development and regulation (Chen, Li et al. 2004, Rodriguez, Vigorito et al. 2007).

MicroRNAs have been shown implicated in many processes of the innate and adaptive immune response. Several examples highlight the importance of microRNAs in the immune response. Transcription of microRNAs such as miR-155 and miR-146a in immune cells is increased in response to inflammatory stimuli such as Toll-like receptor ligands or pro-inflammatory cytokines (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007). Overexpression of miR-21 and miR-196 in bone marrow cells of a murine model blocked granulopoiesis (Velu, Baktula et al. 2009). MiR-155 was shown to be able to increase immature granulocyte numbers and led to physiological granulocyte/monocyte diffusion and growth during Toll like receptors-mediated inflammation (O'Connell, Rao et al. 2008).

Macrophage inflammatory responses involve the up-regulation of several microRNAs such as miR-155, miR-146, miR-147, miR-21 and miR-9 (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007, Bazzoni, Rossato et al. 2009, Liu, Friggeri et al. 2009, Sheedy, Palsson-McDermott et al. 2010). Specific microRNAs such as miR-132, miR-146 and miR-155 can be over expressed by inflammatory mediators including NF-κβ and microbial components and influence the course of inflammation (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007). Many recent studies have shown that miR-155 expression is increased in macrophages in response to stimulation with TNF-α and IFN-β (O'Connell, Taganov et al. 2007, Tili, Michaille et al. 2007). Martinez-Nunez *et al.* found that miR-155 modulates pathogen binding ability by down-regulating expression of DC-specific ICAM3-grabbing non-integrin (DC-SIGN) in human monocyte-derived DCs through suppression of PU.1 expression (Martinez-Nunez, Louafi et al. 2009). MiR-155 directly targets and decreases levels of suppressor of cytokine signalling 1 (SOCS1) and negatively regulates the Toll-like receptor pathway affecting STAT6 pathway (Wang, Hou et al. 2010).

MicroRNAs also play a vital role in the development and function of lymphocytes. MiR-155 seems to regulate T cell and B cell differentiation and function through a complex network of pathways (Rodriguez, Vigorito et al. 2007). Regulatory T cells (T-regs) constitute a subset of $CD4^+$ T cells essential for the maintenance of self-tolerance. The transcription factor Forkhead box P3 (Foxp3) is specifically expressed in T-regs and is a key regulator of their differentiation and suppressor function. Deficiency in Foxp3 results in severe autoimmune disease in both mice and humans (Kim, Rasmussen et al. 2007). MiR-155 maintains T-reg proliferation and homeostasis by down-regulating SOCS1 expression and also directly targets Foxp3 in T-regs contributing to their development (Rodriguez, Vigorito et al. 2007, Kohlhaas, Garden et al. 2009). MiR-146 deficiency in T-regs has also been shown to result in inability to exhibit their suppressor function and IFN-γ response dysregulation through an increase in Signal Transducer and Activator 1 (STAT1) expression and activation (Lu, Boldin et al. 2010). Other microRNAs involved in T cell function modulation are miR-182, miR-126 and miR-326 (Du, Liu et al. 2009, Mattes, Collison et al. 2009, Stittrich, Haftmann et al. 2010).

MiR-150 has been shown to play an important role in the regulation of important mRNAs for pre- and pro-B cell formation or function and can block further development of B cells (Zhou, Wang et al. 2007). Further microRNAs involved in B-cell development and function are miR-155 and miR-181b (Chen, Li et al. 2004, Hu, Fong et al. 2010).

1.6.2 MicroRNAs in inflammatory bowel disease

As described in the previous section, microRNAs have a significant role in processes of the innate and adaptive immune systems. They interact with many aspects thought to contribute to the IBD development such as bacterial sensing through Toll-like receptors, pro-inflammatory cytokines, granulocytes, macrophages and dendritic cells, in addition to playing a role in the adaptive immune response (see section 2.2.1). This makes microRNAs interesting candidates to study as their dysregulation could contribute to augmentation or alleviation of inflammation.

In recent times the main tool for identifying microRNAs involved in specific cellular processes is gene expression profiling using microarray technology. Three types of microarrays in common use are cDNA arrays (purified PCR products of cDNA clones), pre-synthesized oligonucleotides microarrays and in situ synthesized oligonucleotides. For tissue from paraffin blocks specific tissue microarrays (TMA) can be used and tissue from laser micro dissection has been analysed with arrays (Gorreta, Carbone et al. 2012). Microarrays have helped to generate microRNA signatures of disease states. For systematic investigation of microRNA expression, oligonucleotide-based microarrays for microRNAs have been recently developed and several commercial platforms are now available (Liu, Calin et al. 2004).

Microarrays of tissue from IBD patients have characterised the expression profile of microRNAs in CD and UC as compared to the normal distribution in the healthy gut. In one to the first studies using tissue microarrays on IBD samples, Wu *et al.* in 2008 established sigmoid colon biopsy microRNA microarray profiles for healthy control subjects and patients with active UC. Their results showed that three microRNAs (miR-192, miR-375, and miR-422b) were significantly decreased in UC tissues, while eight microRNAs (miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195, and let-7f) were significantly up-regulated in active UC tissues. MiR-192 and miR-21 were the most highly expressed of the active UC–associated microRNAs in human colonic tissues (Wu, Zikusoka et al. 2008).

A further study by the same author investigated microRNAs expression in sigmoid pinch biopsies from 5 CD patients with chronically active CD and 13 control individuals. The results showed that expression of miR-23b, miR-106 and miR-191 was increased in tissues from patients with active

CD, while miR-19b and miR-629 were decreased in CD patients. An assessment of microRNAs expression in terminal ileum biopsies from 6 patients with chronically active terminal ileal CD and 6 control individuals revealed that miR-16, miR-21, miR-22, and miR-594 were overexpressed in chronically active terminal ileal CD tissues (Wu, Zhang et al. 2010).
Fasseu *et al*. investigated the expression of mature microRNAs in inactive colonic mucosa of patients with UC or CD and compared to healthy controls. MicroRNAs with a 5 fold expression difference were considered for data analysis. Fourteen highly altered microRNAs in UC and twenty three in CD were identified in quiescent colonic mucosa, eight being commonly dysregulated in non-inflamed UC and CD (miR-26a,-29a,-29b,-30c,-126*,-127-3p,-196a,-324-3p). Five microRNAs (miR-26a,-29b,-126*,-127-3p,-324-3p) share coordinated dysregulation of expression both in quiescent and in inflamed colonic mucosa of IBD patients. Six microRNAs displayed significantly distinct alteration of expression in non-inflamed colonic biopsies of UC and CD patients (miR-196b,-199a-3p,-199b-5p,-320a,-150,-223). These findings indicate that even in quiescent tissue of IBD patients microRNA balance is disrupted and may identify them as important players in the onset and/or relapse of inflammation from quiescent mucosal tissues in IBD patients (Fasseu, Treton et al. 2010), but some microRNAs elevated less than 5 times may also play an important role in IBD. ***Figure 3*** shows microRNAs dysregulated in IBD (Fasseu, Treton et al. 2010).

All these studies of microRNA expression in IBD tissue have helped to characterise the expression profile of microRNAs in normal, inactive and active IBD tissue, but no consideration of treatments that patients have been taking at the time of the study was noted. Treatments altering the immune response in IBD patients could to have a significant effect on the expression of microRNAs considering their wide range of predicted targets, often involving inflammatory and immunological processes. Influence of medication on microRNA expression in IBD will be part of this thesis.

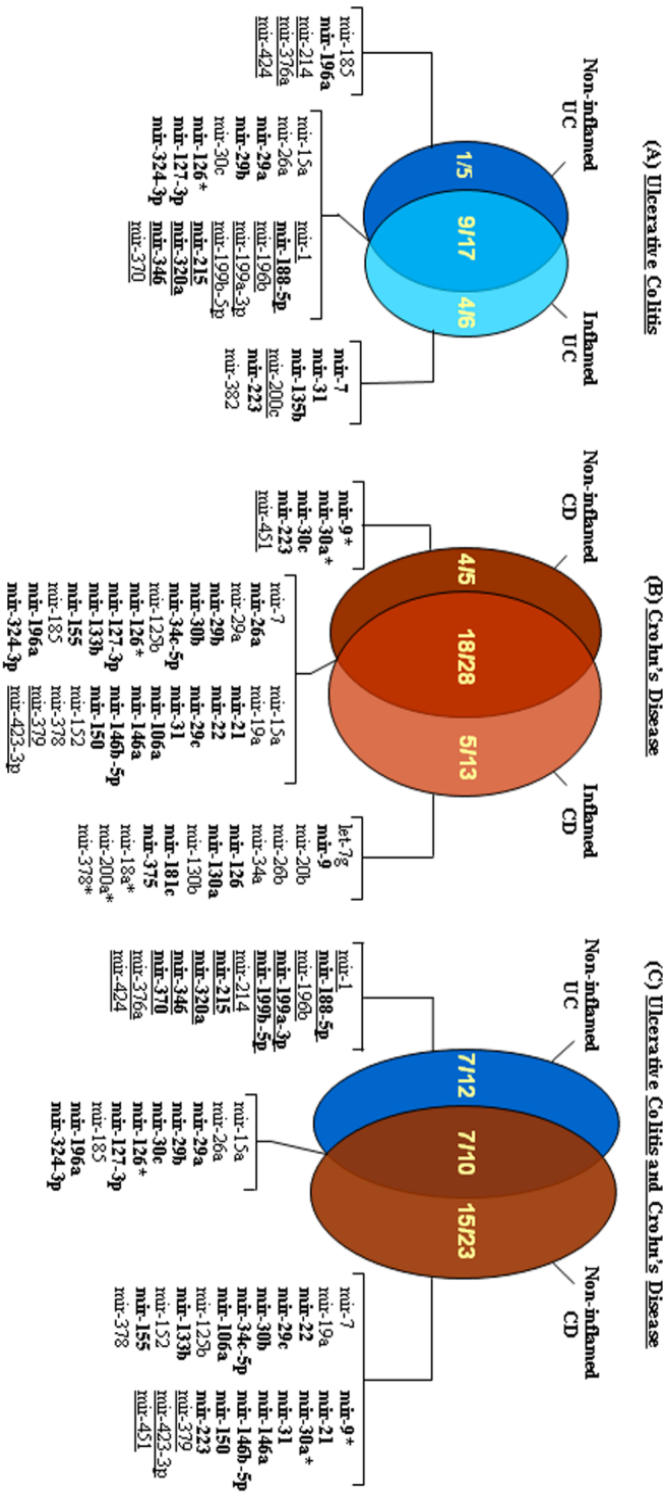


Figure 3. MicroRNAs dysregulated in IBD. Fasseu et al. identified dysregulated microRNAs (5 times up or down-regulated as compared to normal) in non-inflamed and inflamed UC and CD as compared to normal controls. (A) This section shows data for non-inflamed, inflamed and overlapping dysregulated microRNAs and section (B) exhibits the data for CD. Section (C) displays the data for non-inflamed UC and CD and the overlap between the 2. MicroRNAs underlined are down-regulated as compared to normal samples, all others are up-regulated. Adapted from **(Fasseu, Treton et al. 2010).**

1.6.3 MicroRNA-155

MicroRNA-155 (miR-155) is one of the first microRNAs described to be involved in immune function and balance, and probably one of the most described microRNAs in the literature. Mir-155 maps within and is processed from an exon of a non-coding RNA transcribed from the *B-cell Integration Cluster* (BIC) located on chromosome 21 (Lagos-Quintana, Rauhut et al. 2002). MiR-155 has been shown to have multiple functions within the innate and adaptive immune system (Rodriguez, Vigorito et al. 2007) and unsurprisingly has also been shown to be significantly up-regulated in active UC compared with healthy volunteers (Takagi, Naito et al. 2010).

MiR-155 together with miR-146 and miR-132 is significantly involved in innate immunity by regulating the acute inflammatory response after pathogen recognition by Toll-like receptors (TLRs) on monocytes or macrophages (Bakirtzi, Hatziapostolou et al. 2011). Expression of miR-155 was observed to be inducible during bacterial stimulation, as well as after exposure of cells to pro-inflammatory cytokines such as IFN-γ, IFN-β or TNF-α (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007).

There is convincing evidence to indicate that chronic inflammation is a key risk factor for CRC in patients with IBD (Farraye, Odze et al. 2010). NF-κB is a transcriptional factor that regulates a host of genes that are critical to innate and adaptive immunity, cell proliferation, inflammation, and tumour development. Several pro-inflammatory cytokines and chemokines such as TNF-α, IL-1, IL-6, and IL-8 are produced upon NF-κB activation and associated with tumour development and progression (Greten, Manns et al. 2005). A positive correlation between miR-155 up-regulation and NF-κB activation has been reported providing a possible involvement between inflammation and cancer in IBD patients (Ma, Becker Buscaglia et al. 2011).

Martinez-Nunez *et al.* have identified miR-155 as a crucial regulator in human macrophages between the M1 (classically activated, pro-Th(1)) and M2 (alternatively activated, pro-Th(2)) responses through directly targeting IL13Rα1 and reducing levels of IL13Rα1protein. This lead to diminished activation of STAT6 determining the M2 phenotype in macrophages. MiR-155 affected the IL-13-dependent regulation of several genes (SOCS1, DC-SIGN, CCL18, CD23, and Serpine) involved in the establishment of an M2/pro-Th(2) phenotype in macrophages (Martinez-Nunez, Louafi et al. 2011).

The same group also demonstrated how miR-155 participates in the maturation of human dendritic cells and modulates pathogen binding by DC-SIGN through directly targeting the transcription factor PU 1. This suggests a mechanism by which miR-155 regulates proteins involved in the cellular immune response against pathogens (Martinez-Nunez, Louafi et al. 2009).

Increased expression of miR-155 in mature DCs leads to higher levels of IL-12p70 secretion through targeting SOCS1; moreover, over-expression of miR-155 enhances the ability of mature DCs to activate NK cell production of IFN-γ (Lu, Huang et al. 2011). Furthermore, in miR-155-deficient GM-CSF-derived DCs, expression of several pro-inflammatory cytokine genes including IL-12, IL-6, IL-23p19 and IL-23p40 (which are crucial for Th1 and Th17 cell development) were significantly decreased and forced expression of miR-155 in GM-CSF-derived DCs also increased TNF-α production (Zhou, Huang et al. 2010).

T-regs constitute a subset of Foxp3-expressing $CD4^+$ T cells essential for the maintenance of self-tolerance and control of inflammation and therefore play a major role in IBD. MiR-155 maintains T-regs cell proliferation and homeostasis by the down-regulation of SOCS1 expression and also contributes to their development by directly targeting Foxp3 (Rodriguez, Vigorito et al. 2007, Kohlhaas, Garden et al. 2009). MiR-155 has also been shown to be involved in B-cell development and function (Chen, Li et al. 2004, Hu, Fong et al. 2010).

Thus, miR-155 is involved in different inflammatory pathways and in the regulation of innate and adaptive immunity and therefore could be an important player in the pathogenesis of inflammatory bowel disease.

1.6.4 MicroRNA-31

MicroRNA-31 (miR-31) was first identified in HeLa cells (Lagos-Quintana et al. 2001). MiR-31 is located on 9p21.3 and, interestingly, maps to a region close to a cluster of interferon genes (***Figure 4***). This cluster harbours different interferon genes such as Interferon-β-1a (IFN- β1a) which induces a clinical response and remission in patients with UC. IFN-β1a is associated with significant inhibition of IL-13 production and this seems to be facilitated via SOCS proteins (Mannon, Hornung et al. 2011). MiR-31 has been suggested to play an important role in inflammation preventing leucocyte recruitment (Suarez, Wang et al. 2010) and T-reg regulation (Rouas, Fayyad-Kazan et al. 2009) as well as exhibiting multiple functions in neoplastic disease (Schmittgen 2010, Stuelten and Salomon 2010, Olaru, Selaru et al. 2011) .

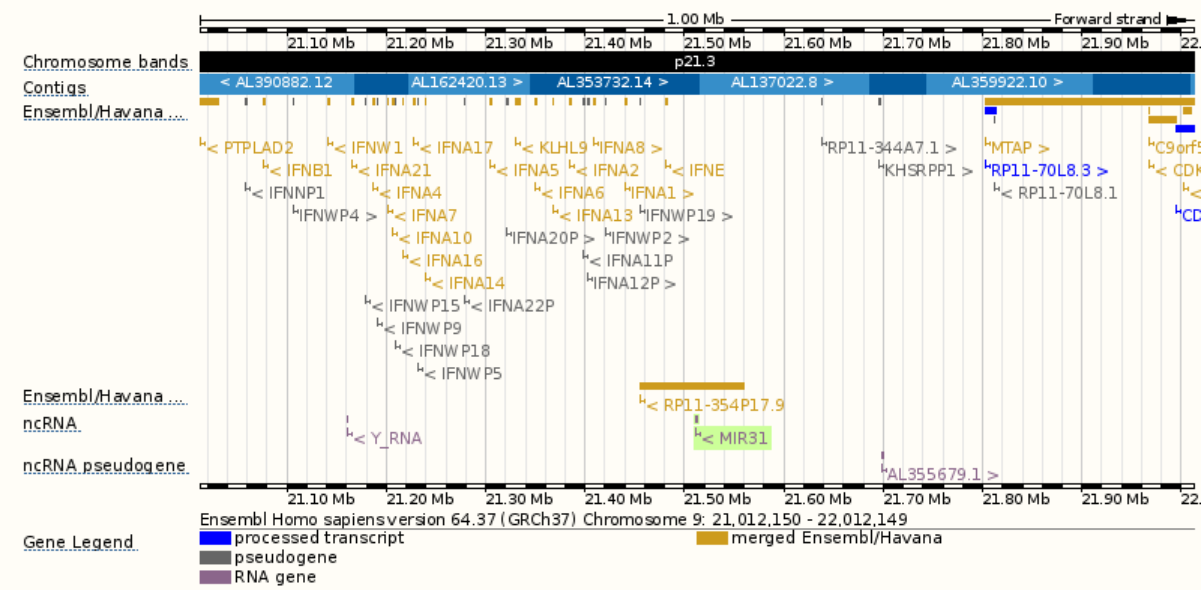


Figure 4. Location of miR-31 on chromosome 9 in the vicinity of the interferon cluster. Obtained from Ensembl (http://www.ensembl.org/Homo_sapiens/Info/Index).

MiR-31 has a wide variety of predicted gene targets such as FOXP3, CD28, HDAC1, HDAC2 and HDAC9 (Rouas, Fayyad-Kazan et al. 2009), highlighting the potential importance of this microRNA and making it a potentially interesting player in IBD. HITS-CLIP is a method increasingly being utilised in conjunction with the analysis of RNA regulation using RNA Seq profiling together with bioinformatic tools, to produce genome-wide maps of sites of RNA regulation which in the future will contribute to identify potiential furher targets of microRNAs (Darnell 2010).

Suarez et *al* reported that TNF-α mediated induction of endothelial adhesion molecules can be regulated by microRNAs that are induced by TNF. Specifically, E-selectin, which recruits neutrophils to be trans-located to a site of inflammation, is targeted by TNF-induced miR-31 and specific antagonism of miR-31 increased neutrophil adhesion to cultured endothelial cells. Conversely, transfections with mimics of these miRNAs decreased neutrophil adhesion to endothelial cells suggesting that miR-31 provides negative feedback control of inflammation and has a potential role in anti-inflammatory therapy (Suarez, Wang et al. 2010).

MiR-31 has been found to be under-expressed in T-reg cells as compared to the levels of this microRNA in other T-cell populations. MiR-31 directly targets the mRNA encoding Foxp3, the master regulator of T-reg cell differentiation and functional activity. This suggests that miR-31 antagonizes a T-reg phenotype by suppressing the expression levels of this important transcription factor (Rouas, Fayyad-Kazan et al. 2009).

MiR-31 has been shown to be up-regulated in inflamed UC (Fasseu, Treton et al. 2010) and a study by Olaru *et al* confirmed this finding. Furthermore, microRNA micro-array analyses of eight chronically inflamed and eight IBD-associated dysplastic rectal tissues identified thirty-two microRNAs that were increased and ten that were decreased in IBD dysplasia. MiR-31 had increased expression in both unaffected and inflamed colonic tissue from IBD patients compared to controls, and miR-31 expression was higher in the affected IBD tissue than the unaffected tissue. No difference in miR-31 expression was found between the IBD dysplasia and IBD carcinoma groups. MiR-31 expression seems to change in a stepwise fashion as tissue goes from normal to chronically inflamed to actively inflamed to neoplastic and could have a role in risk stratifying patients with long term IBD with regards to cancer risk (Olaru, Selaru et al. 2011).

MiR-31 expression was also found significantly increased in sporadic colorectal cancer specimens compared to normal specimens, although miR-31 expression was lower in sporadic colon cancers than in IBD-associated neoplasia. MiR-31 expression levels were able to distinguish IBD-associated neoplasia from normal colonic, unaffected tissue from IBD patients and from inflamed tissue from IBD patients. MiR-31 expression seems to change in a stepwise fashion as colonic tissue goes from normal to chronically inflamed to actively inflamed to neoplastic and could have a role in risk stratifying patients with long term IBD with regards to cancer risk (Olaru, Selaru et al. 2011). Therefore, miR-31 may well play an important role in the link between inflammation and cancer in UC.

MiR-31 has been shown increased in many forms of cancer, but of particular interest is its role in colorectal cancer. Several studies have highlighted the increased expression of miR-31 in colorectal cancer (Slaby, Svoboda et al. 2007, 2009, Brereton, Bodger et al. 2010, Chang, Mestdagh et al. 2010, Earle, Luthra et al. 2010, Iacucci, de Silva et al. 2010, Velayos, Liu et al. 2010, Qualtrough, Smallwood et al. 2011, Ullman and Itzkowitz 2011). Remarkably, it has been shown that suppression of miR-31 with anti-miR increases the sensitivity to 5-FU, a chemotherapy agent, at an early stage, and affects cell migration and invasion in HCT-116 colon cancer cells. Together, these findings highlight the potential role of miR-31 in the therapy of colon cancer (Wang, Stratmann et al. 2010).

Both miR-155 and miR-31 seem to play potentially important roles in IBD, especially in UC and IBD associated colorectal cancer. Moreover, both microRNAs have been shown to directly target IL13Rα1, a crucial player in the pathogenesis of UC. Following these characteristics and given their roles in immunity and oncogenesis, miR-31 and mir-155 were chosen candidates to study in this project.

1.6.5 Other microRNAs predicted to target IL13Rα1

A search of various mircoRNA bioinformatics software and data from previous work in our group for identification of microRNA targets for IL13Rα1 revealed several candidate microRNAs directly targeting IL13Rα1. As mentioned above miR-31 and miR-155 target IL13Rα1, but we have also identified that miR-27ab, miR-148/152, miR-183, miR-196ab, miR-324-5p and miR-374/374ab are predicted to directly target IL13Rα1. Furthermore miR-324-5p and miR-155 were predicted to directly target SOCS1.

In the previously mentioned study by Fasseu *et al* investigating differential expression of microRNAs in IBD in inflamed and inactive samples, a number of our identified microRNAs were found dysregulated such as miR-31 being elevated in inflamed UC and also dysregulated in CD. MiR-196 was down-regulated in UC and miR-148/152, miR-183 and miR-155 were increased in CD, and miR-324 was elevated in UC and CD (Fasseu, Treton et al. 2010). MiR-155 has been shown to be up-regulated in active UC by another study (Takagi, Naito et al. 2010). No reports of dysregulation of miR-27ab and miR-374 in IBD have been published to date.

1.6.6 Potential role of miR-31 and miR-155 in the pathogenesis of UC

MicroRNAs specifically selected in this project are miR-31 and miR-155. MiR-31 has been predicted to target IL13Rα1 by the bioinformatics program TargetScan and miR-155 has been predicted to directly target IL13Rα1 by another bioinformatics program microRNA.org which uses miRanda software (www.microrna.org/microrna/releaseNotes.do) and has been shown by previous work in our lab to directly target IL13Rα1 (Dual Luciferase Reporter system) (Martinez-Nunez, Louafi et al. 2011). MiR-155 has also been shown to target SOCS1, a crucial regulator of the IL-13 pathway (Rodriguez, Vigorito et al. 2007, Kohlhaas, Garden et al. 2009).

MiR-31 and miR-155 have both been shown to be dysregulated in UC (Fasseu, Treton et al. 2010, Takagi, Naito et al. 2010, Olaru, Selaru et al. 2011). UC is driven by a Th2-like response in which NKT cells producing IL-13 (and IL-5) are believed to be the major factor (Strober and Fuss 2011).

STAT-mediated signal transduction plays a pivotal role in IBD inflammatory processes, which are characterized by a mucosal cytokine imbalance (Bouma and Strober 2003, Mudter, Weigmann et al. 2005). UC was found to be associated with increased colonic epithelial STAT6 phosphorylation and STAT6 inhibition with STAT6 siRNA has been shown to prevent IL-13-induced apoptosis and barrier disruption (Rosen, Frey et al. 2011). An Histone De-ACetylase (HDAC) inhibitor has been used to diminish IL-13-induced STAT6 phosphorylation, apoptosis, and claudin-2 expression and it also decreased IL-13-induced reductions in trans-epithelial resistance in HT-29 cells (Rosen, Frey et al. 2011).

Rosen et al also showed that by inducing colitis in a murine model of UC, in STAT6-deficient (STAT6(-/-)) as compared to wild type (WT) mice, colitis was attenuated with improvements in weight, colon length and histopathology implicating that decreased STAT6 phosphorylation plays an important role in preventing epithelial barrier dysfunction and down-regulating Th2-inducing cytokine production (Rosen, Chaturvedi et al. 2013). These findings make STAT6 a possible therapeutic target in UC

Suppressor of Cytokine Signalling 1 (SOCS1), an IL-13 dependent inhibitor of STAT6, is activated through stimulation of IL13Rα1 through IL-4 and IL-13 (Dickensheets, Venkataraman et al.

1999). Type 1 interferons have been shown to influence STAT6 expression via SOCS1 by down-regulation of IL-13 and IL-4 in macrophages (Dickensheets, Venkataraman et al. 1999). Moreover, a clinical study by Mannon P. J *et al* was able to show that Interferon-β-1a induces a clinical response and remission in a large subset of patients with ulcerative colitis that is associated with significant inhibition of IL-13 production and seems to be facilitated via SOCS proteins (Mannon, Hornung et al. 2011). These findings suggest STAT6, SOCS1 and IL-13 may be key players in the pathogenesis of IBD.

The importance of IL-13 in the pathogenesis of ulcerative colitis is highlighted by the fact that anti-IL-13 drugs are currently being evaluated in clinical trials (clinical trial number NCT01284062, Pfizer) ***Figure 5*** demonstrates a simplified cartoon of the role of IL-13 in the pathogenesis of UC and the potential involvement of the microRNAs miR-31 and miR-155 in UC.

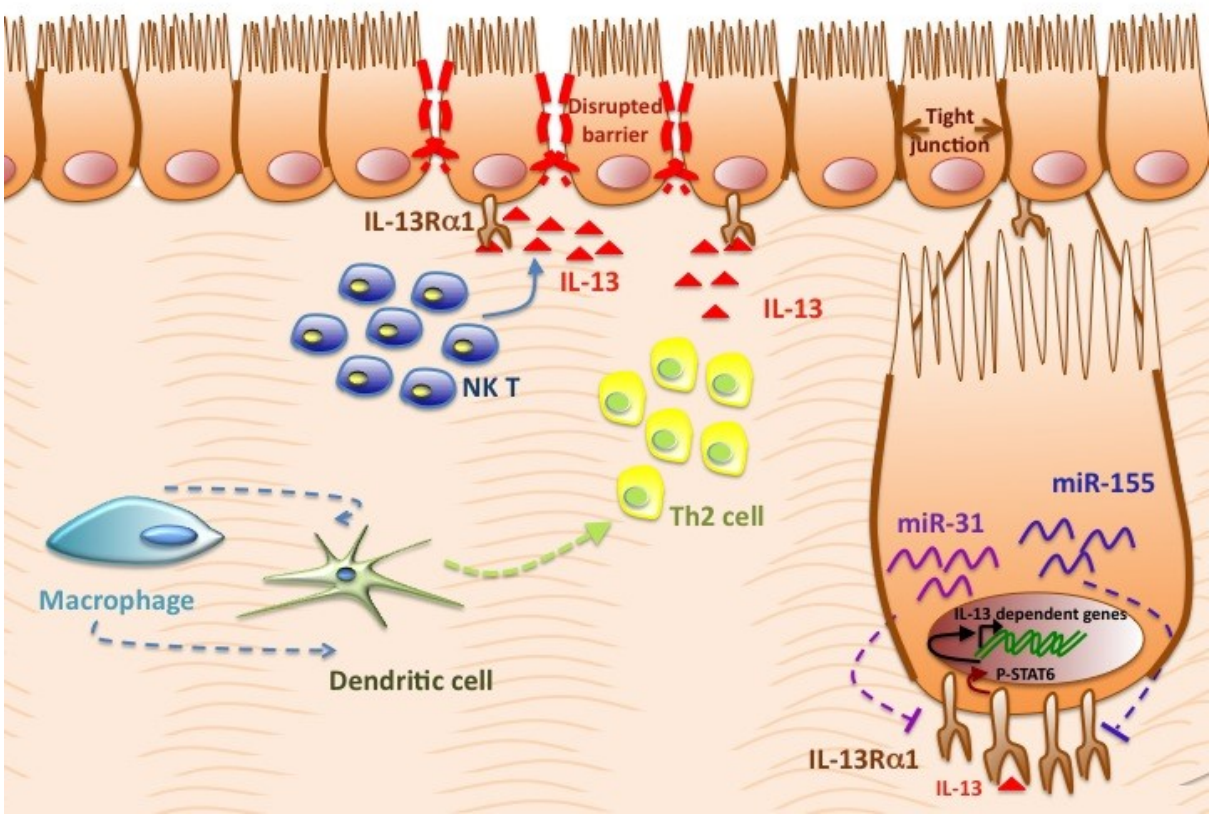


Figure 5 Role of IL-13 in the pathogenesis of UC and the potential involvement of the microRNAs miR-31 and miR-155 in UC.

1.6.7 miR-146a

Despite its role in regulation of the innate and adaptive immune system, miR-146a has not been described to be elevated in inflammatory bowel disease.

Transcription of miR-146a (as well as miR-155) in immune cells is increased in response to inflammatory stimuli such as Toll-like receptor ligands or pro-inflammatory cytokines (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007).

Macrophage inflammatory responses involve the up-regulation of several microRNAs such as miR-155, miR-146a, miR-147, miR-21 and miR-9 (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007, Bazzoni, Rossato et al. 2009, Liu, Friggeri et al. 2009, Sheedy, Palsson-McDermott et al. 2010).

miR-146a and miR-155 can be over expressed by inflammatory mediators including NF-κβ and microbial components and influence the course of inflammation (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007).

MiR-146a deficiency in T-regs has also been shown to result in inability to exhibit their suppressor function and IFN-γ response dysregulation through an increase in Signal Transducer and Activator 1 (STAT1) expression and activation (Lu, Boldin et al. 2010).

miR-146a has been demonstrated to inhibit the expression of IRAK1 and TRAF6, impair NF-κB activity and suppress the expression of NF-κB target genes such as IL-6, IL-8, IL-1β and TNF-α (Taganov, Boldin et al. 2006, (Bhaumik, Scott et al. 2008).

MiR-146a has been shown to be up-regulated in a number of inflammatory auto-immune conditions. Expression of miR-146a was shown to be significantly overexpressed in patients with rheumatoid arthritis in in peripheral blood mononuclear cells and has also been demonstrated to be elevated in patients with systemic lupus erythematosus (SLE) and psoriatic arthritis (Tang, Luo et al. 2009, Chatzikyriakidou, Voulgari et al. 2010, Abou-Zeid, Saad et al. 2011, Pauley and Cha 2011).

The involvement of miR-146a in processes of the innate immune system and its role in autoimmune and inflammatory disease makes miR-146a a very interesting microRNA to explore further in inflammatory bowel disease.

1.7 Effect of therapy in inflammatory bowel disease on cytokine and microRNA expression

Little is known about the effects of medication, frequently used to treat CD and UC, on the expression profile of cytokines commonly up-regulated in IBD.

CD is a mainly Th1 and Th17 mediated process, whereas UC seems to be predominately mediated through Th2 and NK T-cells producing IL-13 (Shih and Targan 2008, Matricon, Barnich et al. 2010, MacDonald, Monteleone et al. 2011, Strober and Fuss 2011).

A comprehensive study, undertaken by Christophi et al, characterised cytokine signalling pathways in the pathogenesis of inflammatory bowel disease (IBD). 70 selected immune genes that are important in IBD signalling were analysed from formalin-fixed, paraffin-embedded colon biopsy samples from normal control subjects and UC and CD patients having either severe colitis or quiescent disease (n = 98 subjects).

Expression levels of signalling molecules including IL-6/10/12/13/17/23/33, STAT1/3/6, T-bet, GATA3, Foxp3, SOCS1/3, and downstream inflammatory mediators such as chemokines CCL-2/11/17/20, oxidative stress inducers, proteases, and mucosal genes were differentially regulated between UC and CD and between active and quiescent disease. A possible role of novel genes in IBD, including SHP-1, IRF-1,TARC, Eotaxin, NOX2, arginase I, and ADAM 8 was also documented (Christophi, Rong et al. 2012). This extensive study did not take different medications patient were taking into account and was performed on paraffin fixed specimens as opposed to fresh samples. Nevertheless, it gives a comprehensive cytokine signalling profile in IBD mucosal biopsies.

Current drug treatments in IBD aim to induce and maintain the patient in remission and ameliorate the disease's secondary effects. The influence of IBD treatments on cytokine signalling pathways has not been well described in the literature and therefore the influence of medication on these pathways is largely unknown.

Generally, medications used in the treatment of IBD are 5-ASA (Amino Salicylates) preparations such as mesalazine (mainly in UC) (Camma, Giunta et al. 1997), topical and systemic steroids (Ford, Bernstein et al. 2011), the frequently used immune modulators such as the thiopurines 6-

mercaptopurin and azathioprine (Fraser, Orchard et al. 2002). Anti-TNF-α agents such as Infliximab and Adilumimab have given new options of treatment in the last few years (D'Haens, Panaccione et al. 2011).

Multiple mechanisms of action have been demonstrated for 5-ASA compounds in vitro such as inhibition of the production of pro-inflammatory cytokines such as IL-1, TNF-α and induction of lymphocyte apoptosis (Doering, Begue et al. 2004), inhibition of the lipoxygenase pathway, scavenging of free radicals and oxidants as well as inhibition of the inflammatory-related NF-κB pathway (Barnes and Karin 1997, Liptay, Bachem et al. 1999). Another proposed mechanism of action of 5-ASA is via inhibition of IL-2 production in peripheral mononuclear cells inhibiting T-cell proliferation, changing cell adhesion expression pattern, reducing antibody production and mast cell release, and interfering with macrophage and neutrophil chemotaxis (Fujiwara, Mitsui et al. 1990).

The direct effect of 5-ASA compounds on pro-inflammatory and pro-fibrotic mediators in IBD biopsies are limited and the microRNA expression in colonic biopsies in patients with IBD remain unknown.

The involvement of miR-31, miR-146 and miR-155 in T-cell biology and inflammatory pathways affected by 5-ASA compounds may well influence the expression of these microRNAs in patients who are taking 5-ASA drugs for colitis.

6-Mercaptopurine and its pro-drug azathioprine (AZA) are purine analogues which interfere with nucleic acid synthesis, exhibit anti-proliferative effects on activated lymphocytes and, most recently, have been shown to induce apoptosis in T-cells and epithelial cells (Tiede, Fritz et al. 2003, Schroll, Sarlette et al. 2005). It has also been shown that thiopurines selectively inhibit inflammatory gene expression in activated T-lymphocytes (Thomas, Myhre et al. 2005).

Data on the effect on the cytokine expression in colonic inactive and active IBD biopsies of patients on thiopurines is limited and the microRNA profile of inflammatory bowel disease patients who are taking thiopurines has not been determined to date. Considering the involvement of miR-31, miR-146 and miR-155 in T-cell biology, thiopurines may have a profound effect on the expression of these microRNAs in IBD patients on azathioprine or 6-mercaptopurine.

Infliximab and Adalimumab are anti-TNF-α monoclonal antibodies that bind with high specificity and affinity to human TNF-α (Hanauer, Feagan et al. 2002, Rutgeerts, Sandborn et al. 2005, Colombel, Sandborn et al. 2007, Reinisch, Sandborn et al. 2011).

Infliximab has been shown to neutralize soluble TNF-α (Van Den Brande, Peppelenbosch et al. 2002). Ringheanu et al found that Infliximab reduced the expression of TNFα, IL-1β , IL-6, and IL-8 mRNA in PBMC isolated from patients before and 1 h after INF infusion (Ringheanu, Daum et al. 2004). Activated T cells isolated from colonic biopsies of patients with CD cultured with Infliximab showed decreased expression of IFN-γ (Agnholt and Kaltoft 2001).

TNF-α is of vital importance in the pathogenesis of UC and CD and has been shown to induce both miR-31 and miR-155 expression (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007, Suarez, Wang et al. 2010). Transfection of miR-146a into THP-1 monocytes could reduce expression of IRAK1/TRAF6 and decrease TNFα expression (Pauley and Cha 2011).

TNF-α is a direct target in IBD therapy with anti-TNF-α therapy such as Infliximab and Adalimumab. Such biologic therapies may therefore have a profound influence on the expression of microRNAs involved in the TNF-α pathway in IBD tissues. The effect of anti-TNF agents used in IBD therapy on the expression of microRNAs is unknown.

Ljung et al reported a decreased immune-histochemical expression of TNF-α, IL-1β and INF-γ in mucosal biopsies in patients with Crohn's disease after treatment with infliximab (Ljung, Axelsson et al. 2007). Down-regulation of MMPs was demonstrated in CD biopsies of patients treated with Infliximab, with absence of this effect in non-responders (Di Sabatino, Saarialho-Kere et al. 2009).

IFX induced down-regulation of the mucosal TNF-α and IFN-γ mRNA expression in UC patients. The numbers of T lymphocytes and macrophages were significantly decreased in patients with endoscopically healed mucosa after IFX treatment (Olsen, Cui et al. 2009).

Investigating the effect of IBD therapy on pathways deregulated in IBD and the expression of microRNAs involved in vital processes of inflammatory pathways and T-cell pathology could give a new insight in treatment choice and may be able to predict treatment failure.

2 Materials and Methods

2.1 Equipment

7900HT Fast Real-Time PCR System (#4329001, Applied Biosystems, California, USA)

DNA Engine TETRADTM 2 Peltier Thermal Cycler (#TA001175, Esco Technologies, Inc., USA)

Luminometer Model TD-20/20 (#2-1796-CE, Turner BioSystems)

MagNA Lyser Instrument (#03358976001, Roche Products Ltd., Hertfordshire, UK)

Nanodrop (Fisher Scientific UK Ltd, Leicestershire, UK)

Thermo Scientific Heraeus FRESCO 17 Centrifuge (#CFH-203-010K, Fisher Scientific UK Ltd, Leicestershire, UK)

2.2 List of reagents

Agarose (#BPE 1356-100 C4, Thermo Fisher Scientific Inc, Leicestershire, UK)

Albumin from bovine serum ≥98% (BSA) (#A7906-100G, SIGMA-ALDRICH COMPANY LTD., Dorset, UK)

Anti-beta Actin antibody - Loading Control (ab8227, Abcam plc., Cambridge, UK)

Anti-IL-13Rα1 (sc-25849, Santa Cruz Biotechnology Inc., Heidelberg, Germany)

Anti-miR™ miRNA Inhibitors—hsa-155 (#AM17000, Ambion, Texas, USA)

Anti-miR™ miRNA Inhibitors—hsa-31 (#AM17000, Ambion, Texas, USA)

Anti-miR™ miRNA Inhibitors—Negative Control #1 (#AM17010, Ambion, Warrington, UK)

Anti Phospho STAT6 (#9361, Cell Signalling Technology- New England Biolabs (UK) Ltd., Hertfordshire, UK)

Anti STAT6 (#9362, Cell Signalling Technology- New England Biolabs (UK) Ltd., Hertfordshire, UK)

AQIX® RS-I 1x kit (#RSI/KIT, Aquix Ltd., UK)

BCA Protein Assay Kit (#23225, Thermo Fisher Scientific Inc, Leicestershire, UK)

Beta-Mercaptoethanol (#6250, SIGMA-ALDRICH COMPANY LTD., Dorset, UK)

Bromophenol blue (#114405-25G, SIGMA-ALDRICH COMPANY LTD., Dorset, UK)

Complete Protease Inhibitor Cocktail Tablets (#0469312400, Roche Products

Ltd., Hertfordshire, UK)

D-MEM Media - GlutaMAX™-I (#61965-059, GIBCO®, In vitrogen Ltd., Paisley, UK)

D-sorbitol (#85529-250G SIGMA-ALDRICH COMPANY LTD., Dorset, UK)

Dual-Luciferase Reporter Assay System (#E1910, Promega UK Ltd, Hampshire, UK)

EDTA Disodium Salt (#D/0700/53 C30, Thermo Fisher Scientific Inc, Leicestershire, UK

Ficoll-Paque™ PLUS (#17-1440-03, GE Healthcare)

Foetal Bovine Serum (Heat Inactivated) (#10108-165, Life Technologies LTD, Paisley, UK)

Genopure Plasmid Maxi Kit (#03143422001, Roche Products Ltd., Hertfordshire, UK)

Glycerol (#G/0650/08 C35, Thermo Fisher Scientific Inc, Leicestershire, UK)

Glycine (#G/0800/60 C40, Thermo Fisher Scientific Inc, Leicestershire, UK)

Glycogen 20mg (#10901393001, Roche Products Ltd., Hertfordshire, UK)

GM-CSF (#11343128, Immunotools, Germany)

High Capacity cDNA Reverse Transcription Kit (#4374967, Applied Biosystems, USA, California)

IGEPAL CA-630 (#I7771-50ML, SIGMA-ALDRICH COMPANY LTD., Dorset, UK)

Immobilon polyvinylidene difluoride membrane (#IPVH00010, Millipore Ltd..,

Watford, UK)

INTERFERinTM (# 409-01, Polyplus-transfection SA, France)

LB Agar (#L2897 SIGMA-ALDRICH COMPANY LTD., Dorset, UK)

LB Broth (#L3022 SIGMA-ALDRICH COMPANY LTD., Dorset, UK)

LDH-Cytotoxicity Assay Kit II (#: K313-500, BioVision, Inc. California, USA)

LS Columns (#130-042-401, Miltenyi Biotech, Germany)

MicroRNA Primers were purchased from Life Technologies Ltd., Paisley, UK

NaCl for molecular biology (#71376-1KG, SIGMA-ALDRICH COMPANY LTD., Dorset, UK)

NuPAGE® MOPS SDS Running Buffer (for Bis-Tris Gels only) (20x) (#NP0001, In vitrogen Ltd, Paisley, UK)

NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm, 10 well (#NP0321BOX, In vitrogen Ltd, Paisley, UK)

NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm, 15 well (#NP0323BOX, In vitrogen Ltd, Paisley, UK)

Opti-MEM® I Reduced-Serum Medium (1X), liquid (#31985-062, GIBCO ®)

PBS tablets (#18912-014, GIBCO ®, In vitrogen Ltd, Paisley, UK)

Pefablock SC (#11585916001, Roche Products Ltd., Hertfordshire, UK)

Pfu DNA Polymerase 100U (#M7741, Promega UK Ltd, Hampshire, UK)

Polyclonal Goat Anti-Rabbit Immunoglobulin HRP conjugated (#P0448, Dako UK Ltd., Cambridgeshire, UK)

QIAquick Gel Extraction Kit (#28704, Qiagen, Sussex, UK)

Recombinant human IL-13 (#213-IL-005, R&D Systems Europe Ltd., Oxfordshire, UK)

Restriction enzymes used were purchased from New England Biolabs (Herts, UK)

RPMI Medium 1640 - GlutaMAX™-I (#72400-054, GIBCO®, In vitrogen Ltd, Paisley, UK)

SmartLadder (#MW-1700-10, Eurogentec Ltd., Hampshire, UK)

Sodium dodecyl sulphate (#S/5200/53 C107, Thermo Fisher Scientific Inc, Leicestershire, UK)

Spectra™ Multicolor Broad Range Protein Ladder Lot 34744 (#SM1841, Fermentas)

Sucrose (#84097-1KG, SIGMA-ALDRICH COMPANY LTD., Dorset, UK)

TaqMan® Universal PCR Master Mix, No AmpErase® UNG (#4364341, Applied Biosystems, USA, California)

TOPO® TA Cloning® Kit (with pCR®2.1-TOPO®) with One Shot Mach1™-T1R

TRI Reagent Solution (#AM9738, Applied Biosystems, Warrington, UK)

Tris Base Ultra Pure (#BPE152-1 C131, Thermo Fisher Scientific Inc, Leicestershire, UK)

Tris hydrochloride 1M pH 7.5 (#BPE1757-500, Thermo Fisher Scientific Inc, Leicestershire, UK)

TRIzol (#15596-018, In vitrogen Ltd, Paisley, UK)

Tween 20, Molecular Grade: 500ml (#41116134, Promega UK Ltd, Hampshire, UK)

Ultrapure DNAse⁄RNAse-Free distilled water (#10977035, Invitrogen Ltd., Paisley, UK)

2.3 Bioinformatics search for microRNAs targeting IL13Rα1

IL13Rα1 has been identified as a direct target of miR-155 by our group. MiR-155 directly targets IL13Rα1 and reduces levels of IL13Rα1 protein. This leads to diminished activation of STAT6 and determines the M2 phenotype in human macrophages (Martinez-Nunez, Louafi et al. 2011).

There are several bioinformatics tools available on line to predict microRNA-target interactions. Microrna.org (http://www.microrna.org/microrna/home.do) is an on line tool for the identification of microRNA-target predictions and expression profiles (John, Enright et al. 2004). It is based mostly in known rules for microRNA-target interactions and conservation. Using microRNA.org miR-31, miR-196 and miR-324 were identified to target IL13Rα1. ***Figure 6*** shows the predicted binding sites for miR-31 and miR-155 in the 3'UTR of IL13Rα1.

Using the microRNA search tool TargetScan (www.TargetScan.org), which emphasises conservation amongst species to predict microRNA-target interactions (Lewis, Burge et al. 2005, Garcia, Baek et al. 2011), revealed several candidate microRNAs for IL13Rα1 3'UTR targeting. We found miR-31, miR-27a/b, miR-148/152, miR-183 and miR-374a/b as possible microRNAs directly targeting IL13Rα1. Furthermore miR-324 was predicted to target SOCS1 and mIR-155 has been demonstrated to directly target SOCS1 mRNA (Lu et al., 2009). ***Figure 7*** shows the predicted microRNAs for IL13RA1 3'UTR binding according to Target Scan.

An additional bioinformtatics tool is the PITA algorithm which allows predicting microRNA-target interactions (Kertesz, Iovino et al. 2007) by on line searching of possible microRNAs targeting a specific input sequence (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html). All microRNAs previously mentioned were predicted to target IL13Rα1 3'UTR.

In the previously mentioned study by Fasseu *et al* investigating differential expression of microRNAs in IBD in inflamed and inactive samples miR-31 was elevated in inflamed UC and also dysregulated in CD. In addition miR-148/152, miR-183 and miR-155 were increased in CD, and miR-324 was elevated in UC and CD. The cut off in this study in the micro-array was an up or down-regulation of at least 5 fold (Fasseu, Treton et al. 2010). Moreover, miR-155 has been shown to be up-regulated in active UC by another study (Takagi, Naito et al. 2010). No reports of dysregulation of miR-27a/b and miR-374 in IBD were found in the literature.

MiR-31 was thought to be a strong candidate for further experiments due to its proven dysregulation in UC (Fasseu, Treton et al. 2010) and its major role in the inflammation and colorectal cancer axis in UC (Olaru, Selaru et al. 2011). Interestingly, miR-31 predicted binding site on the IL13Rα1 3'UTR is proximal to that of miR-155 (***Figure 6***), making it an interesting prospect to investigate a possible collaboration or even synergy between miR-31 and miR-155.

MiR-155 has been proven to directly target IL13Rα1 mRNA by our group (Martinez-Nunez, Louafi et al. 2011) and has also been shown to be elevated in active UC (Takagi, Naito et al. 2010). These observations make miR-155 an attractive microRNA for further studies.

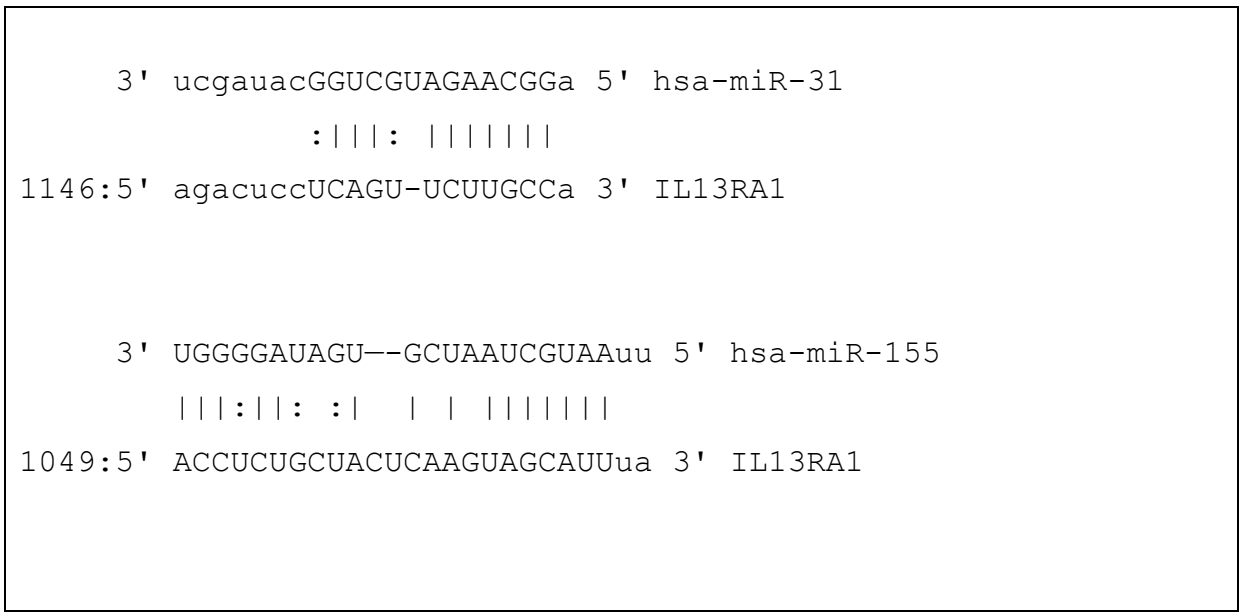


Figure 6. Schematic interaction of miR-31 and miR-155 with IL13Rα1 3'UTR.

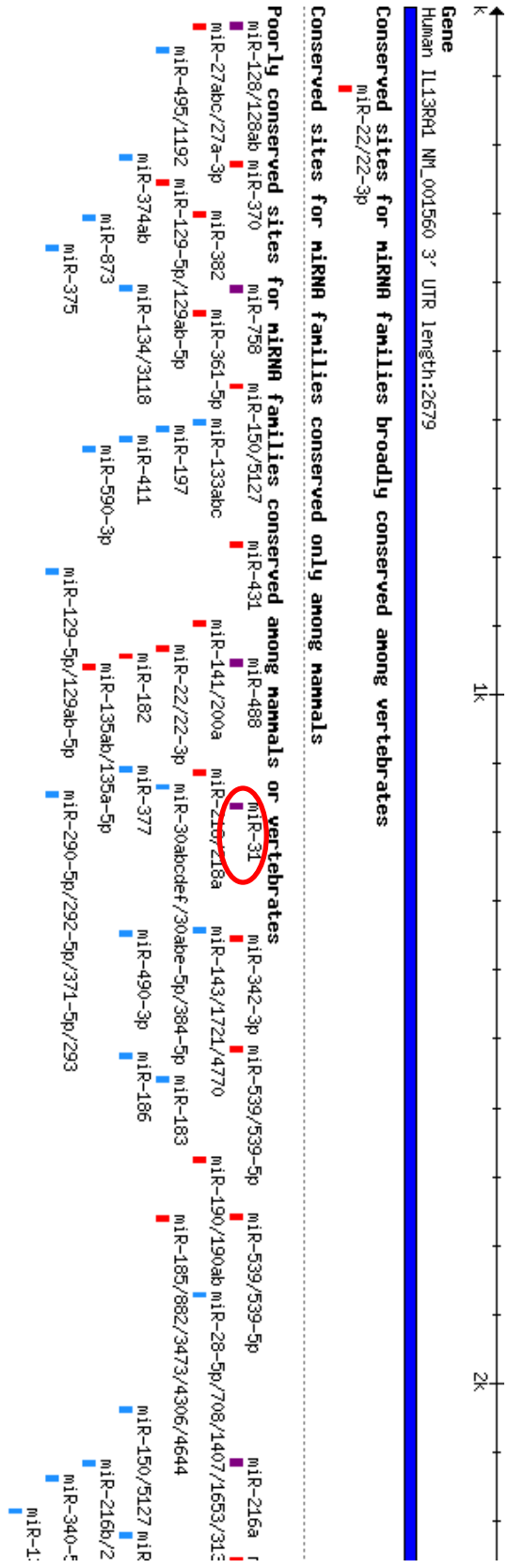


Figure 7. MicroRNA targets on TargetScan for IL13Rα1. Adapted from http://www.targetscan.org/.

2.4 Ethics approval and Consent

This study was given a favourable ethical opinion for conduct in the NHS by the Southampton and South West Hampshire Research Ethics Committee (A) (Reference Number 10/H0502/69).

Ethics approval was granted for taking up to eight pinch biopsies in total from one patient as well as taking blood samples. Informed consent was obtained from all patients participating in the project according to ethics guidelines.

2.5 Sample collection and clinical characteristics of patient population

We recruited patients who gave informed consent according to ethics guidelines. Consecutively over 18 months all patients with colonic IBD (both UC and CD) and healthy controls, who attended a weekly IBD endoscopy list or healty controls who agreed to have biopsies taken in line with the ethics approval, had 8 biopsies taken from unaffected (quiescent disease) and inflamed areas (active disease) in the sigmoid colon and matched with biopsies from healty controls. All patient samples were frozen with liquid nitrogen and stored at -80 degrees in a tissue bank for use in experiments. In patients with distal disease 4 biopsy samples from the affected area were collected and further four paired biopsies were taken from the inactive proximal colon of each patient. Data was generated from a single biopsy of one patient, or in case of paired samples from a single biopsy of the inactive and active segment.

For explant culture experiments UC 8 samples from active UC (endoscopic Mayo score 2-3) were taken from sigmoid colon for immediate transfection experiments.

Demographic data was recorded including age, sex, diagnosis, duration and extent of disease. Current drug history was carefully noted. In patients with UC a full clinical and endoscopic Mayo score was calculated and in patients with CD a Harvey-Bradshaw index and the Simple Endoscopic Score for Crohn's Disease (SES-CD) was noted.

In all experiments mRNA was normalised with GAPDH, microRNA with RNU44 and Western Blots were normalised with Actin.

2.5.1 Demographic data for paired UC samples

11 patient samples (available samples at the time of experiment) with distal disease were identified from our tissue bank who had an endoscopic Mayo score of 2-3 in the active segment and a corresponding biopsy (Mayo score 0-1) from the inactive sigmoid area and matched with 11 healthy controls samples from the sigmoid colon as a baseline control. For demographic data of the study population see ***table 7***.

	UC-paired samples 11	**Control 11**
Average Age	**47.3** (range 22-85)	**56.1** (range 46-78)
Sex	Male **6** Female **5**	Male **6** Female **5**
Diagnosis	UC	Normal colon
Duration of disease	Years **10.0** (range 1-34)	n/a
Extent of disease	Left sided colitis **6** Distal colits **5**	n/a
Mayo score	Clinical **7.55** (STDEV 2.12) Endoscopic **2.73** (STDEV 0.47)	0
5-ASA	**6**	n/a
Thiopurines	**3** (2 also on 5-ASA)	n/a
Infliximab/Adilumimab	**1** (also on 5-ASA)	n/a
No medication	**3**	n/a

Table 7. Demographic data of the study population.

2.5.2 Demographic data for western blotting experiments in UC

For western blotting samples were collected from age matched normal controls on no medication having colonoscopies for polyp follow up (N=6), 6 from individual patients with inactive UC (Mayo score 0-1) and 6 from individual patients with active UC (Mayo score 2-3). Sample number of 6 for each sample group was chosen to be able to demonstrate 2 complete Western blot membranes with each group represented on the same blot. The demographic data for western blotting experiments is summarized in ***Table 8.***

	UC inactive (N=6)	**UC active (N=6)**
Average Age	42.3 (range 23-69)	40.1 (range 20-72)
Sex	Male: 3/Female: 3	Male: 3/Female: 3
Diagnosis	Inactive UC	Active UC
Duration of disease	Years 12.6 (range 1-36)	Years 14.4 (range 1-31)
Extent of disease	Left sided colitis 5 Distal colits 1	Pan-colitis 3 Left sided colits 3
Endoscopic Mayo score	0.5 (STDEV0.5)	2.5 (STDEV0.5)
5-ASA	2	2(6)
Thiopurines	2 (2 also on 5-ASA)	2 (2 also on 5-ASA)
No medication	3	3

Table 8. Demographic data of the study population for western blotting.

2.5.3 Demographic data for epithelial cell isolation

For epithelial cell isolation samples were collected from normal age matched controls on no medication having colonoscopies for polyp follow up (N=5), 5 from individual patients with inactive UC (Mayo score 0-1) and 5 from individual patients with active UC (Mayo score 2-3). 5 biopsies from each patient were use for the epithelial isolation to achieve a good RNA yield. Demographic data is summarized in ***Table 9.***

	UC inactive (N=5)	**UC active (N=5)**
Average Age	39.3 (range 23-71)	42.1 (range 21-67)
Sex	Male: 3/Female: 2	Male: 2/Female: 3
Diagnosis	Inactive UC	Active UC
Duration of disease	Years 10.6 (1-34)	Years 12.2 (range 1-41)
Extent of disease	Left sided colitis 4 Distal colits 1	Pan-colitis 3 Left sided colits 2
Endoscopic Mayo score	0.4 (STDEV0.5)	2.6 (STDEV0.5)
5-ASA	2	2
Thiopurines	1 (1 also on 5-ASA)	1 (1 also on 5-ASA)
No medication	2	2

Table 9. Demographic data of the study population for epithelial cell isolation.

2.5.4 Demogaphic data for pre-microRNAs transfections

For transfection of active UC biopsy explant cultures with pre-miR-31 and pre-miR-155 and its combination we initially recruited 15 patients. 8 patient data was discarded due to unacceptable GAPH (mRNA) normalising gene C_T values, possibly due to degradation of RNA following culture of explants for 24 hours. ***Table 10*** shows the demographic data of the 7 patients analysed.

	Active UC
Average Age	47.3 (range 22-81)
Sex	Male: 4/Female: 3
Diagnosis	Active UC
Duration of disease	Years 11.6 (range 1-39)
Extent of disease	Left sided colitis 3 Pancolitis 4
Mayo score	Clinical 7.88 (STDEV 2.12) Endoscopic 2.64 (STDEV0.47)
5-ASA	3
Thiopurines	2 (2 also on 5-ASA)
Infliximab/Adilumimab	0
No medication	2

Table 10. Demographic data of the study population for pre-microRNAs transfections.

2.5.5 Demographic data for UC patients on IBD medication

For the analysis of mRNA and microRNA expression in UC patients and the effect of drug treatment (Chapter 10, Results VII) we recruited 28 patients with inactive and 28 patients with active UC. ***Table 11*** summarizes the demographic data employed.

	Inactive UC	**Active UC**
Average Age	43.9 (range 19-81)	41.5 (range 22-78)
Sex	Male: 15/Female: 13	Male: 14/Female: 14
Diagnosis	Inactive UC 28	Active UC 28
Duration of disease	Years 8.9 (range 1-24)	Years 12.0 (range 1-32)
Extent of disease	Left sided colitis 12 Pancolitis 16	Left sided colitis 13 Pancolitis 15
Mayo score	Clinical 1.78 (STDEV 1.49) Endoscopic 0.54 (STDEV0.51)	Clinical 9.72 (STDEV 1.39) Endoscopic 2.64 (STDEV0.47)
No medication	9	8
5-ASA	10	8
Thiopurines	9 (2 also on 5-ASA)	12 (2 also on 5-ASA)

Table 11. Demographic data of the population with UC for miRNA and mRNA profiling and the effect of drug therapy.

2.5.6 Demographic data for Crohn's patients under medication

For the analysis of mRNA and microRNA expression in Crohn's patients and the effect of drug treatment (Chapter 11, Results VIII) we recruited 23 patients with inactive and 23 patients with active CD. ***Table 12*** summarizes the demographic data employed.

	Inactive CD	**Active CD**
Average Age	33.6 (range 21-66)	39.0 (range 19-64)
Sex	Male 11/Female 12	Male 12/Female 11
Diagnosis	Inactive CD 23	Active CD 23
Duration of disease	Years 10.2 (range 1-32)	Years 10.5 (range 1-29)
Extent of disease	Ileocolonic 11 CD colitis 12	Ileocolonic 10 CD colitis 13
The Harvey-Bradshaw index	Clinical 3.26 (STDEV 1.32)	Clinical 9.91 (STDEV 2.06)
SES-CD	0	1.78 (STDEV0.73)
No medication	7	7
Thiopurines	9	8
Biologics	7	8

Table 12. Demographic data of the population with Crohn's disease for miRNA and mRNA profiling and the effect of drug therapy.

2.6 Endoscopy and sample collection

A specific endoscopy list has been dedicated for the surveillance and assessment of patients with IBD by Dr Cummings (Consultant Gastroenterologist and Clinical Lead of IBD Services at the Southampton University Hospital). Up to eight samples were collected at the time. Samples were either frozen in liquid nitrogen or used for explants cultures and stored in Aquix RS-1 medium.

2.7 Snap freezing of samples

Samples were approximately 2mm-5mm in size (maximum diameter of biopsy forceps 9mm), transferred into cryovails (coded and dated), snap frozen in liquid nitrogen and later stored at -80 degrees.

2.8 Cell culture

2.8.1 THP-1 cells

THP-1 cells are a human leukemic cell line cultured from the blood of a boy with acute monocytic leukaemia (Tsuchiya, Yamabe et al. 1980). IL-13 signalling through IL13Rα1 activating STAT6 has previously been shown in THP-1 cells (Hart, Bonder et al. 1999).

This cell line was kindly provided by Dr. Christopher Pickard (ATCC number TIB-202™). It was maintained in RPMI with 10% Foetal Calf Serum (FCS) at a cell density of 5-7x10^5 cells/mL.

2.8.1.1 Cell number determination in THP-1 cells for seeding in a 24 well plate

THP-1 cells were taken from a T 75 flask. Cells were counted (number of cells/ml) and seeded at 50.000, 100.000 and 150.000 in triplets.

The distribution of cells was assessed the next day and the distribution of 100.000 cells/ well in a 24 well plate was determined as being ideal for the future work.

2.8.1.2 IL-13 dose response experiment in THP-1 cells

THP-1 cells were cultured in a T 75 flask in RPMI with 10% RPMI. Cells were counted and 100.000 cells were seeded in duplicates per 24 well plate in 500µl RPMI with 10% FCS.

2 wells were used as control with no additional substance. 2 wells were stimulated with IL-13 at 25ng/ml, 2 wells were stimulated with IL-13 at 50ng/ml and 2 wells were stimulated with IL-13 at 100ng/ml. One well of each concentration was harvested at 1h, 8h and 24h. Cells were centrifuged at 1200rpm for 5 minutes and were collected with TRIzol and frozen at -80C.

2.8.2 HT-29 cells

HT-29 cells are a cell line derived from a human colon adenocarcinoma and exhibit characteristics of normal intestinal epithelium such as epithelial polarity, presence of the actin binding protein villin and the occurrence of an enterocytic differentiation (Chantret, Barbat et al. 1988). HT-29 cell lines have been used *in vitro* for experiments in IBD in relation to IL-13 (Heller, Fromm et al. 2008, Mandal and Levine 2010). HT-29 cells express IL-4Rα, IL13Rα1, and IL-13Rα2 shown by RT-PCR (Blanchard, Durual et al. 2004).

This cell line was cultured in DMEM plus 10% FCS at a cell density of 5-7·105 cells/mL cells and passed when confluent.

2.8.2.1 Cell number determination in HT 29 cells for seeding in a 24 well plate

HT 29 cells were detached from a T 75 flask using 2ml of trypsin and re-suspended in DMEM plus 10% FCS. Cells were counted (number of cells/ml) and seeded at 15.000, 30.000, 60.000 and 90.000 in triplets.

The distribution of cells was assessed the next day and the distribution of 60.000 cells/ well in a 24 well plate was determined as being ideal for the future work achieving 75% confluence in a 24 well plate.

2.8.2.2 IL-13 dose response experiment in HT 29 cells

HT 29 cells were detached from a T 75 flask using 2ml of trypsin and re-suspended in DMEM plus 10% FCS. Cells were counted (number of cells/ml) and 60.000 cells re-suspended in 1 ml/well of DMEM plus 10% FCS and seeded in duplicates. Cells were left overnight to attach.

2 wells were used as un-stimulated controls. 2 wells were stimulated with IL-13 at 25ng/ml, 2 wells were stimulated with IL-13 at 50ng/ml and 2 wells were stimulated with IL-13 at 100ng/ml. One well of each concentration was harvested at 1h, 8h and 24h. After removal of media, the

wells were washed with PBS 3 times and then 300μl of TRIzol were applied. Cells were collected with TRIzol and frozen at -80C.

2.8.3 HeLa cells

HeLa cells are one of the oldest human cell lines and are derived from cervical cancer cells taken on February 8, 1951 (Berlanga, Bradshaw et al. 2011). HeLa cells were used for transfections in the Dual Luciferase experiments.

2.8.4 Monocyte derived macrophages

Monocytes were isolated from human peripheral blood mononuclear cells (PBMCs). PBMCs were obtained from healty donor's blood by separation using centrifugation in a density gradient of Ficoll-Paque^{TM} PLUS (GE Healthcare). Blood samples were diluted in PBS by half before subjecting them to density gradient separation. Diluted samples were carefully pipetted on top of Ficoll in a ratio 1:4 (Ficoll: sample) approximately. These gradients were centrifuged at 2000 rpm 20min at room temperature with no centrifuge brake. The layer of separated PBMCs was then removed using a serological pipette and washed 4 times in PBS to remove cell debris and platelets. Centrifugation during washes was at 1200 rpm 10 min at room temperature. PBMCs were then labelled using CD14 magnetic beads (Miltenyi) in MACS buffer (approximately 80μl and 20μl CD14-beads per 10^8 PBMCs) at 4°C during 18min inside a 15ml facon tube. After labelling, cells were washed in MACS buffer and pelleted at 1200 rpm during 10min. CD14+ cells (monocytic fraction) were separated by positive selection using magnetic LS MACS columns (Miltenyi) and the column was washed 4-6 times with MACS buffer to remove unlabelled cells. Isolated monocytes were plated at a cell density of 10^6 cells/ml onto 96 well plates (U bottom) and maintained in RPMI 10% FBS supplemented with 500U/ml GM-CSF to allow macrophage differentiation.

2.8.5 Explant cultures

Four sigmoid biopsies from each active UC patient with a Mayo score greater than 2 were taken and transferred into separate 1.5mL tubes containing Aquix solution to preserve them. Once in the lab, samples were transferred onto a 96 well plate (U-bottom) in 200μL of Aquix solution.

Whole biopsies containing mucosa (epithelial cells) and submucosa (cells of innate and adaptive immune system/fibroblasts) were then transfectediRs as explant cultures with pre-miRs (see ***2.10.4***).

2.9 Transfections

2.9.1 Transfection of HT- 29 cells with anti-microRNAs.

HT-29 cells were detached from a T 75 flask using 2ml of trypsin and re-suspended in DMEM plus 10% FCS. Cells were counted (number of cells/ml) and 60.000 cells re-suspended in 1 ml of DMEM plus 10% FCS, seeded in couplets in a 24 well plate and left to attach overnight. 2 control wells were transfected with 100nM of anti-miR-control with 5μl of Interferin and 2 wells each were transfected with anti-miR-31 or anti-miR-155 at 100nM and 5μl of Interferin. Transfection protocol was as follows (quantities are per well of a 24 well plate):

- Cell medium was replaced with 500μl of fresh medium/well.
- DMEM/Anti-microRNA mixture was prepared: 100mM of anti-miR-control, anti-miR-31 or anti-miR-155 was added to 100μl of serum free DMEM, vortexed for 15 seconds and then let to rest for 5 minutes at room temperature
- 5μl Interferin were added to the DMEM/Anti-microRNA mixture, vortexed for 15 seconds and then let to rest for 5 minutes at room temperature before a final vortex for 15 seconds. The mix was left at room temperature for 15 minutes.
- Interferin/anti-miR mixture was pipetted onto the appropriate well to make a total volume of 600μl gently shaking the plate to ensure a good distribution within the well. Cells were incubated under regular culture conditions (37°C and 5%CO_2).

Cells were harvested at 24 and 48h. After removal of media, the wells were washed with PBS 3 times and then 300μl of TRIzol were added. TRIzol samples were left at room temperature for 5min and frozen at -80C.

2.9.1.1 Transfection of HT-29 cells with pre-miR-31 and pre-miR-155 in different concentrations to assess effect on IL-13 dependant genes

HT 29 cells were detached from a T 75 flask using 2ml of trypsin and re-suspended in DMEM plus 10% FCS. Cells were counted (number of cells/ml) and 60.000 cells re-suspended in 1 mL of DMEM plus 10% FCS and seeded in triplets in a 24 well plate. Cells were left to attach overnight. DMEM with 10% FCS was replaced with 500µl of fresh medium. 3 control wells each were transfected with 100nM of pre-miR-control with 5µl of Interferin and 3 wells each were transfected with 100nM of pre-miR-31 or pre-miR-155 or a combination of 50nM of a pre-miR-31/155 mix all with the addition of 5µl of Interferin and incubated under regular culture conditions for 24 hours. Transfection was performed as described in ***2.11.1***.

After 24 hours (transfection protocol was established by our group in earlier published work) cells were harvested and collected in 300µl of TRIzol for RNA extraction (see 3.15), reverse transcribed and qPCR was performed as in 3.16 and 3.17, respectively, to detect expression of SOCS1, CCL18, TNF-α, MMP-9, Serpine and SMAD2.

2.9.1.2 Transfection of HT-29 cells with pre-/anti-miR 31 and 155 separately and in combination with selective IL-13 stimulation

HT 29 cells were detached from a T 75 flask using 2ml of trypsin and re-suspended in DMEM plus 10% FCS. Cells were counted (number of cells/ml) and 60.000 cells re-suspended in 1 ml of DMEM plus 10% FCS were seeded in triplets in a 24 well plate and left to attach overnight. DMEM with 10% FCS was replaced with 500µl of fresh medium. Twice three control wells each were transfected with 100nM of anti-miR-control or pre-miR-control with 5µl of Interferin and 3 wells each were transfected with 100nM of anti-miR-31 or anti-miR-155 or pre-miR-31 or pre-miR-155 or a combination of 50nM of a pre-miR-31/155 mix or 50nM of anti-miR-31/155 all with the addition of 5µl of Interferin and incubated under regular culture conditions for 24 hours. Transfection was done following the transfection protocol described in 3.10.1.
After 24 hours half the wells were stimulated with 100ng/ml IL-13 and cells were harvested after one hour in 300µl of TRIzol for RNA extraction. RNA was extracted (3.15), reverse

transcribed and qPCR was performed as in 3.16 and 3.17, respectively, to detect changes in SOCS1 expression.

2.9.2 Transfection of THP-1 cells with pre- miR 31 and 155 separately and in combination +/- IL-13 stimulation

THP-1 cells were cultured in a T 75 flask in RPMI 10% FCS. Cells were counted and 10^5 cells were seeded in triplicates per 24 well plate in 500μl RPMI with 10% FCS.

Each well was transfected with 100nM of pre-miR-control, 100nM of pre-miR-31, 100nM pre-miR-155 or a combination of 50nM of a pre-miR-31/155 mix as in ***2.10.1*** and incubated under regular culture conditions. After 24 hours half the wells were stimulated with IL-13 100μM. Cells were harvested after one hour and collected in 300μl of TRIzol for RNA extraction. RNA was isolated (see ***2.16***), reverse transcribed and qPCR was performed as in ***2.17*** and ***2.18***, respectively.

2.9.3 Transfection of primary macrophages

Isolated monocyes from healthy donors (see ***2.10.4***) were plated onto 96 well plate (U bottom) in the presence of 500U/ml of GM-CSF (Immunotools) to induce macrophage differentiation. Cells were transfected by passive uptake or natural phagocytosis with pre-miR-31 (100nM), pre-miR-155 (100nM) and its combination (50nM of pre-miR-31 and pre-miR-155 each) for 48 hours and then every other sample was stimulated with IL-13 at 100ng/ml for 24 hours.

2.9.4 Transfection of explant cultures

Sigmoid biopsies were transferred onto a 96 well plate (U-bottom) in 200μL of Aquix solution and transfected with 100nM of pre-miR-control, 100nM pre-miR-31, 100nM pre-miR-155 or a combination of 50nM pre-miR-31 and pre-miR-155 using the same protocol as in ***2.10.1***. Explants were then incubated under regular conditions (37C and 5%Co2) during 24h and then harvested for RNA isolation and RT-qPCR (see ***sections 2.16, 2.17*** and ***2.18***, respectively)

2.10 Cloning

2.10.1 Vectors generated

pCDNA3.1_miR-31: This vector containing the coding region of microRNA-31 was cloned amplifying the genomic region of miR-31 by PCR from genomic DNA and then cloned using TOPO TA Cloning Kit (Invitrogen) into pCR4 vector following manufacturer's instructions. This clone was named pCR4_miR-31. The primers used were miR-31_FOR: (XhoI) CTCGAG CAC TGA AGA GTC ATA GTA TTC TCC and miR-31_REV: (HindIII) AAGCTT AAA TCC ACA TCC AAG GAA GGG CGC. These primers were designed to amplify miR-31 between XhoI and HindIII restriction sites at its 5' and 3' end, respectively. The annealing temperature employed was 60°C. Clones were verified by sequencing (Geneservice, Oxford). pCR4_miR-31 was then digested with XhoI/HindIII and the detached fragment was sub-cloned into pCDNA3.1 (-) XhoI/HindIII digested plasmid. Ligation was performed overnight at 16°C using T4 DNA Ligase High Concentrated (NEB) following manufacturer's instructions. Clones were verified by sequencing (Geneservice, Oxford). ***Figure 8*** shows a schematic of miR-31 cloning into pCDNA3.1 (-).

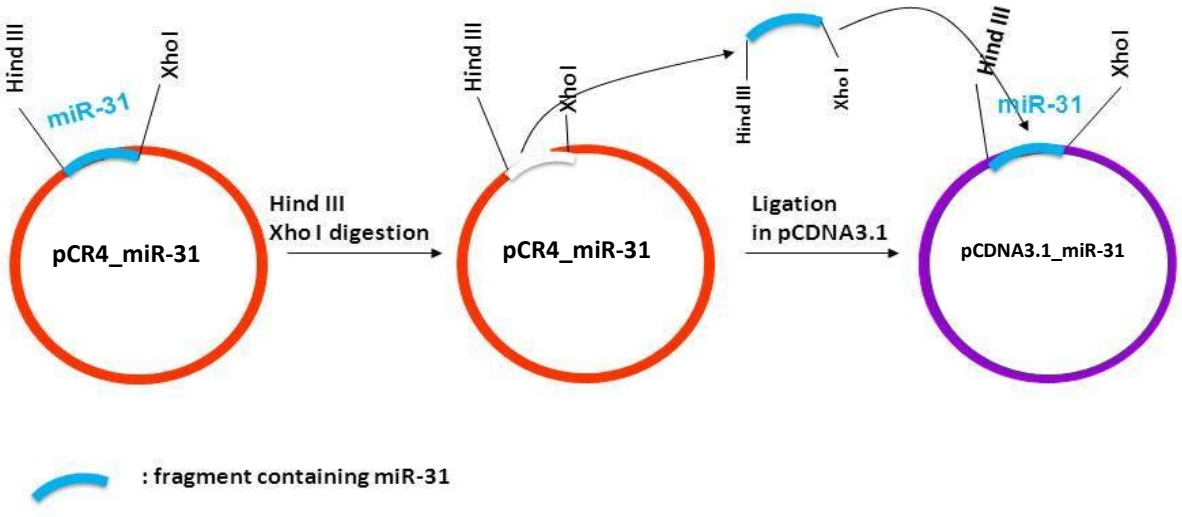


Figure 8. MiR-31 expression vector construct. The genomic region encompassing miR-31 was cloned into pCR4 TOPO vector. pCR4_miR-31 was subsequently digested with Hind III/Xho I enzymes and the excised fragment was cloned into pCDNA3.1. (Adapted from Martinez-Nunez).

2.10.2 Site directed mutagenesis of pRLTK_WT_IL13RA1_3'UTR for the binding site of miR-31

pRLTK_WT_IL13RA1_3'UTR was previously generated by our group (Martinez-Nunez, Louafi et al. 2011). TargetScan prediction of miR-31 binding site in IL13Rα1 3'UTR corresponded to position 1146 (***Figure 5***). **pRLTK_WT_IL13RA1_3'UTR** was used to perform site directed mutagenesis of the predicted binding site for miR-31 using QuickChange Site Directed Mutagenesis (Stratagene) following the manufacturer's instructions to create **pRLTK__MUT1_3´UTR_IL13RA1** by altering the binding site of miR-31. (***Figure 9***). Primers used were **IL13RA1_3'UTR_MUT1_FOR** : CTG CTA CTC AAG TCG GTA CCA CTG TGT CTT TGG TTT GTG CTA GGC CCC and **IL13RA1_3'UTR_MUT1_REV:** GGG GCC TAG CAC AAA CCA AAG ACA CAG TGG TAC CGA CTT GAG TAG CAG.. Primers were designed following manufactures' instructions as follows:

- both primers harboured the same mutation and annealed in opposite strands of the sequence flanked by 10-15nt of correct sequence on each side;

- primer length was within 25-45nt;

- primers meting temperature (T_m) must be greater than or equal to 78°C using the formula $T_m = 81.5 + 0.41(\%GC)-675/N - \%$ mismatch, where "%GC" is the percentage of guanosine+cytosines in the primer and "N" is number of nucleotides in the primer.

- GC content must be at least 40% and primers should preferentially terminate in on or more G or C bases and primers.

Figure 10 shows a schematic of the site-directed mutagenesis protocol.

```
       3'     ucgauacGGUCGUAGAACGGa 5' hsa-miR-31
                     :|||: |||||||
1146:  5'     agacuccUCAGU-UCUUGCCa 3' WT IL13RA1

       3'     ucgauacGGUCGUAGAACGGa 5' hsa-miR-31
                     :|||: ||||||
1146:  5'     agacuccUCAGU-UCGGUUUa 3' MUT1 IL13RA1
```

Figure 9. Binding site of miR-31 in IL13Rα1 (WT) and the mutant version (MUT1) generated by site directed mutagenesis of pRLTK__WT_3´UTR_IL13RA1 into pRLTK__MUT1_3´UTR_IL13RA1, respectively.

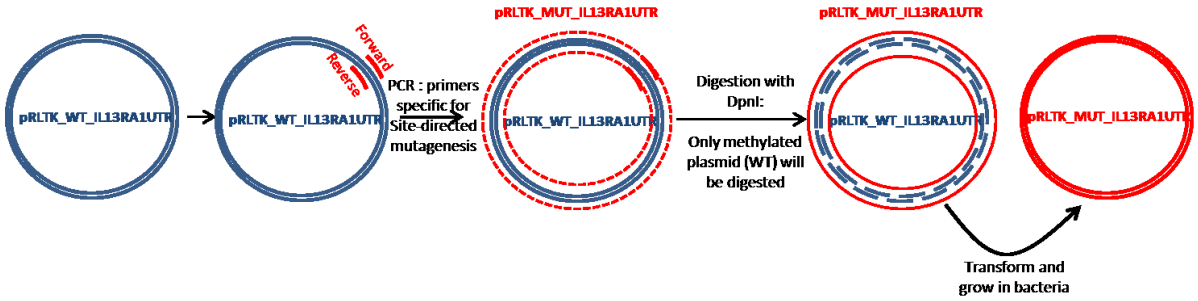


Figure 10. Schematic of the protocol for site directed mutagenesis (Adapted from Martinez-nunez).

2.10.3 Other vectors used

pCDNA3.1.BIC (miR-155), pRLTK_WT_IL13RA1_3'UTR, pRLTK__MUT1_3´UTR_IL13RA1 (mutant for binding site 1 of miR-155) and **pRLTK__MUT2_3´UTR_IL13RA1** (mutant for binding site 2 of miR-155) were previously generated in our group as described (Martinez-Nunez, Louafi et al. 2009, Martinez-Nunez, Louafi et al. 2011). **pGL3** (Promega) containing Firefly luciferase was used as normalizer in Dual-Luciferase experiments (see 3.15).

Vectors used and generated in this study are listed in ***Table 13*** and primers used in ***Table 14***.

	Vector name	Clone	Vector function
miR-31/155	**pCR4_Bic (miR-155)**	BIC- pri-miR-155	Cloning
	pCDNA3.1.BIC (miR-155)	BIC- pri-miR-155	Expression
	pCR4_ miR-31	pri-miR-31	Cloning
	pCDNA3.1._miR-31	pri-miR-31	Expression
IL13RA1	**pCR2.1._3'UTR_IL13RA1**	WT 3'UTR IL13RA1	Cloning
	pRLTK _WT_IL13RA1_3'UTR	WT 3'UTR IL13RA1	Reporter
	pRLTK__MUT1_3´UTR_IL13RA1(miR-155)	Mutant1 3'UTR IL13RA1**(miR-155)**	Reporter
	pRLTK__MUT2_3´UTR_IL13RA1(miR-155)	Mutant1 3'UTR IL13RA1**(miR-155)**	Reporter
	pRLTK__MUT1_3´UTR_IL13RA1(miR-31)	Mutant1 3'UTR IL13RA1**(miR-31)**	Reporter

Table 13. List of vectors generated during this study. From left to right, first column shows the gene these constructs relate to. Second column shows the name of the vector; third column shows the cloned product and fourth column the function or use of the vector (Adapted from Martinez-Nunez).

	Name	Sequence
miR-31	**miR-31_FOR**	CTCGAG CAC TGA AGA GTC ATA GTA TTC TCC
	miR-31_REV	AAGCTT AAA TCC ACA TCC AAG GAA GGG CGC
miR-155	**BIC_FOR**	CTC GAG TAT GCC TCA TCC TCT GAG TGC
	BIC_REV	AAG CTT ACG AAG GTT GAA CAT CCC AGT GAC C
3'UTR IL13RA1	**IL13RA1_3'UTR_FOR**	GGC TGT TAG GGG CAG TGG AG
	IL13RA1_3'UTR_REV	CAG AGC CTT GGC TGG CTG G
	IL13RA1_3'UTR_MUT1_FOR (miR-155)	CTG CTA CTC AAG TCG GTA CCA CTG TGT CTT TGG TTT GTG CTA GGC CCC TTT GTG CTA GGC CCC
	IL13RA1_3'UTR_MUT1_REV(miR-155)	GGG GCC TAG CAC AAA CCA AAG ACA CAG TGG TAC CGA CTT GAG TAG CAG
	IL13RA1_3'UTR_MUT2_FOR(miR-155)	CCA TGT GAG GGT TTT CAG GGC CGA TAT TTG TGC ATT TTC TAA ACA G
	IL13RA1_3'UTR_MUT2_REV(miR-155)	CTG TTT AGA AAA TGC ACA AAT ATC GGC CCT GAA AAC CCT CAC ATG G
	IL13RA1_3'UTR_MUT1_FOR (miR-31)	CTG CTA CTC AAG TCG GTA CCA CTG TGT CTT TGG TTT GTG CTA GGC CCC
	IL13RA1_3'UTR_MUT1_REV (miR-31)	GGG GCC TAG CAC AAA CCA AAG ACA CAG TGG TAC CGA CTT GAG TAG CAG

Table 14. List of primers used in this study. From left to right, first column shows the gene amplified by these primers; second column shows the name of the primer and third column the primer sequences (Adapted from Martinez-Nunez).

2.11 Dual Luciferase system

The Dual Luciferase Reporter (DLR) system is an *in vitro* tool used to investigate the translational consequences of non-protein coding sequences. Two different reporter genes are employed, Renilla and Firefly luciferase. The assay exploits the differing biochemical requirements for luminescence of the firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) luciferase proteins allowing sequential measurement of both luciferase activities in a single assay (Sherf 1996). One is used as reporter and the other functions as a normaliser.

Firefly luciferase luminescence occurs via the oxidation of luciferin to oxyluciferin in a reaction requiring ATP, Mg^{2+}, and O_2. The luminescence generated by *Renilla* luciferase utilizes O_2 and coelenterazine (Sherf 1996). The two genes catalyse the conversion of different substrates in a chemical reaction that leads to the emission of light of different wavelengths which can be measured (represented in ***Figure 11***).

Using the DLR reagents (Promega), the luminescence of the firefly luciferase reaction can be measured by adding luciferin as substrate, and this reaction is subsequently quenched while simultaneously activating the luminescence of the *Renilla* luciferase. Therefore sequential measurement of the luminescence of both (normalizer and reporter) in a single reaction tube is possible (Sherf 1996). Translational rate is measured as light emission readout captured by a luminometer.

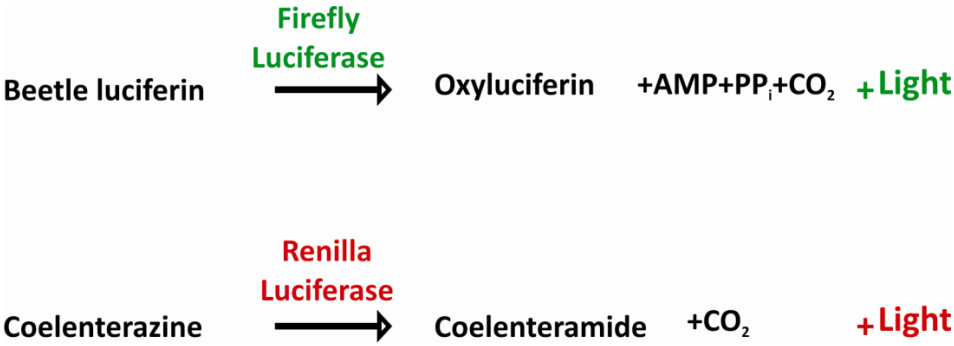


Figure 11. Dual Luciferase Reporter enzymatic reactions. Firefly Luciferase (green) and Renilla Luciferase (red) are two enzymes that can be both used as reporter and normaliser genes. These enzymes catalyse the conversion of different substrates (beetle luciferin and colenterazine) with the emission of light of different wavelengths, represented as green and red colours. Light emission changes are captured by a luminometer and serve as readout of the enzyme quantity in each of the reactions (Adapted from Martinez-Nunez).

To assay post transcriptional regulation like the one exerted by microRNAs, sequences are cloned at the 3'end of the reporter gene. If microRNAs directly target that specific 3'UTR sequence, co-transfection of the microRNAs will lead to a diminished expression of the reporter clone with the 3'UTR of interest. The Dual-Luciferase Reporter Assay System (Promega) was used to determine the direct targeting of IL13RA1 3'UTR by miR-31 and miR-155 (see section 2.12 for constructs cloning). The assay is summarized in ***Figure 12***. Renilla luciferase was used as reporter gene for assaying miR-155 and miR-31 effects and Firefly luciferase was used as normalizer.

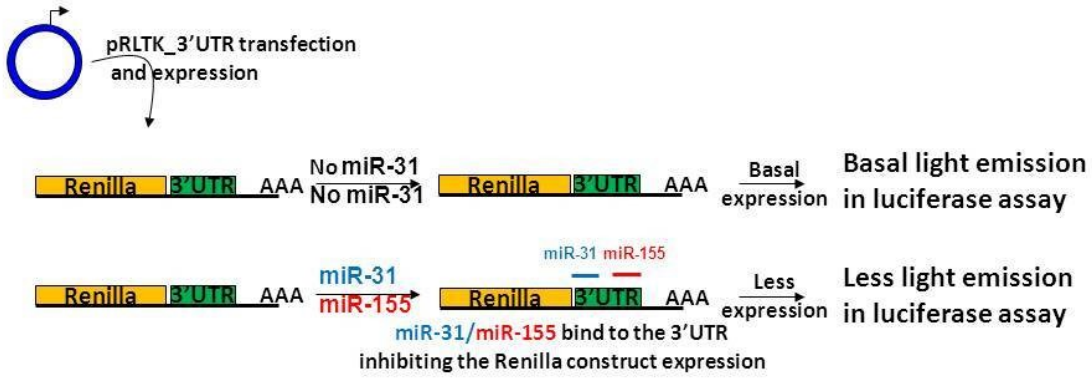


Figure 12. Luciferase system representation for mirR-31 and miR-155 directly targeting of IL13rα1 3'UTR. Renilla luciferase reporter vector (blue) containing Renilla gene ("Renilla" in red) fused to the IL13rα1 3'UTR ("3'UTR" in pink) is co-transfected with or without MiR-31 and miR-155 or a mix of both over expressing vector (indicated as "MiR-155/MiR-31" in green). The absence of miR-31/155 over expression (upper reaction) leads to a basal expression of the construct with the concomitant light emission in the luciferase enzymatic assay. The over expression of miR-31/155 (lower reaction) inhibits the expression of the Renilla construct leading to a decrease in light emission in the luciferase enzymatic reaction (Adapted from Martinez-Nunez).

2.12 Plasmid transfections: Direct targeting of IL13RA1 by miR-31 and miR-155

Plasmid DNA preparations were isolated and purified using Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions.

The constructs **pRLTK_WT_3´UTR_IL13RA1**, **pRLTK_MUT1_3´UTR_IL13RA1** (mutant site 1 for miR-155 binding) or **pRLTK_MUT2_3´UTR_IL13RA1** (mutant site 2 for miR-155 binding) and **pRLTK_MUT1_3´UTR_IL13RA1** (mutant site for miR-31) were co-transfected with **pCDNA3.1.BIC**, **pCDNA3.1._miR-31** or **pCDNA3.1** (-) empty vectors into HeLa cells employing LT1 (Mirus) according to manufacturer's instructions. Normalization was achieved by co-transfecting pGL3 (Promega). Assays will be measured with the Dual-Glo kit (Promega). Experiments were performed in triplicates. Statistical differences were determined using Student's t test and GraphPad Prism, Prism 5 version 5.00 for Windows software.

2.13 Isolation of epithelium from biopsies.

Isolation of epithelial cells from colonic samples was done in Epithelial Isolation Buffer (EIB). This protocol was established by Dr Jane Collins, Senior Lecturer. Previous control experiments showed high purity of epithial cells (epithelial cell markers) in the isolate with minimal contamination by immune cells. (Personal communication from Dr Jane Collins).

The composition of EIB (pH 7.3) was: 27mM Trisodium citrate, 5mM Na_2HPO_4, 96mM NaCl, 8mM KH_2PO_4, 1.5mM KCl, 0.5mM DTT, 55mM D-Sorbitol and 44mM Sucrose. RNAse inhibitor (400U/mL) and phosphatase inhibitors were freshly added to the buffer every time.

Five colonic biopsies from the same patient taken from an area in the sigmoid colon were snap frozen in liquid nitrogen and then transferred into 1mL of EIB at 4°C in a cold room on dry ice. Samples were inverted gently by hand ten times until the solution became cloudy indicating the detachment of the epithelial cells from the biopsies. The solution was again shaken by hand fifteen times. To detach cells from the crypts samples were gently vortexed 3 times for 10 seconds to free intact or partially broken crypts from the underlying matrix. Samples were then spun at 4°C at 3000rpm for 5min. Pelleted cells (epithelium) were resuspended in TRIzol and stored at -80°C or processed immediately as in ***2.15***.

2.14 RNA isolation

RNA was isolated from different sources:

- tissue samples frozen at -80C;
- straight away at the end of the explant culture experiments;
- frozen TRIzol samples kept at -80C for processing at a later date.

RNA was extracted using TRIzol (Invitrogen) following manufacturer's instructions and modifying some of the steps. Cells or tissue samples were mixed with 300μl of TRIzol and left at room temperature for 5 min. In the case of frozen tissue samples these were mixed with 300μl of TRIzol, disrupted with the MagNA Lyser Instrument and left at room temperature for 5 min. 200 μl of chloroform per mL of TRIzol were added, vortexing each sample for at least 15 seconds and leaving them for a further 5min at room temperature. Samples were then centrifuged at 13000 rpm in a bench minifuge at 4°C for 30 min to separate the organic and aqueous phases. The aqueous phase containing the RNA was carefully pipetted off and the RNA was mixed with 180μl of isopropanol and glycogen (1μg/mL) by vortexing 15 secs, left at room temperature for 10 minutes and then left 15 minutes at -80C to further precipitate the RNA. After centrifugation at 4°C at 13000rpm for 30 minutes, the pellet was washed with ice-cold 75% ethanol and left on ice for 15 minutes. Samples were then centrifuged at 13000rpm for further 15 minutes at 4°C. Ethanol was removed and the pellets were left to air dry. RNA pellets were then re-suspended in RNAse free water (GIBCO) and the amount of RNA was quantified using a Nanodrop. Quality of RNA was confirmed with the nanodrop aiming for a 260/280 ratio of 1.95-2.05 for all samples used. RNA was used immediately for reverse transcription and the remaining RNA was kept at -80°C.

2.15 Reverse Transcription and Real Time PCR

2.15.1 mRNA reverse transcription

RNA reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer´s instructions. 500ng of total RNA were used in the reverse transcription reaction using Random Hexamer primers. The reverse transcription protocol employed was as follows:

10min at 25°C

2h at 37°C

5min at 85°C

Hold at 4°C

cDNA samples were then kept on ice until use for qPCR and then stored at -20°C.

2.15.2 MicroRNA Reverse Transcription

For the detection of microRNAs, 10ng of total RNA were used with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with specific stem loop primers for microRNAs miR-31, miR-27a, miR-152, miR-155, miR-183, miR-196, miR-324 or miR-374. RNU44 was used as housekeeper control. All primers were from TaqMan® MicroRNA Assays (Applied Biosystems) following manufacturer's instructions.

30min at 16°C

30min at 42°C

5min at 85°C

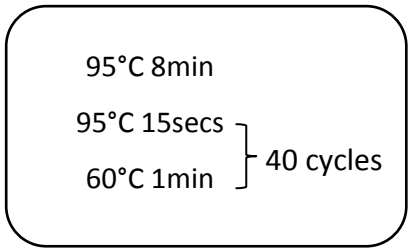
Hold at 4°C

Samples were then kept on ice until use for qPCR and then stored at -20°C.

2.16 Real Time PCR

2.16.1 mRNA Real Time PCR

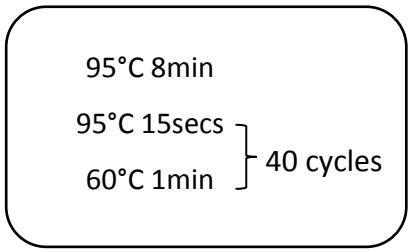
Real Time PCR for mRNA was performed following manufacturer's instructions using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems). mRNA levels were detected using TaqMan® Gene Expression Assays (Applied Biosystems). Reactions were carried out in duplicates in a final volume of 5μL per well on 384 well plates. The protocol for qPCR employed was:



Data was analysed with SDS 2.4 from Applied Biosystems using ΔΔCt method. This method relies on C_T values from the target gene and normalizer. These must have been amplified with primers of approximately the same efficiency. Our primers were commercially standardized and checked (Life Technologies). C_T stands for Cycle Threshold, this is, the cycle number at which fluorescence in the PCR reaction reaches a fixed threshold. Later values mean late amplification, so lower levels of the specific gene. The maximum number of cycles in the qPCR reaction was set at 40, a standard used in this reaction. GAPDH was used as a normaliser.

2.16.2 microRNA Real Time PCR

Real Time PCR for microRNAs was performed using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) following manufacturer's instructions. microRNAs and RNU44 were detected using Applied Biosystems TaqMan® MicroRNA Assays following manufacturer's instructions. The protocol for qPCR employed was:



Data was analysed with SDS 2.4 from Applied Biosystems using $\Delta\Delta C_t$ method (***see 2.18.1***).

2.17 Western Blotting

Cells were lysed in NP-40 1% and Complete Protease Inhibitor Cocktail (Roche). Protease inhibitors were added in the moment of cell lysis. Lysis was performed on ice during 15 min, and cell lysates were centrifuged at 13000 rpm in a top bench centrifuge at 4°C. Supernatants containing whole cell extracts were collected and quantified using the BCA Assay (Pierce, Thermoscientific) following manufacturer´s instructions. 30μg of cell lysates were mixed with 6x sample loading buffer (Invitrogen) to a final concentration of 1x. Beta-Mercaptoethanol was added in a 1:50 ratio to ensure reducing conditions and samples were boiled during 5min before subjected to SDS-PAGE. Electrophoresis and transfer of the samples was done using precasted gelsfrom Invitrogen:NuPAGE®Novex 4-12% Bis-TrisGel 1.0mm, 10 well in the manufacturer's electrophoresis and transfer system XCell SureLock® Mini-Cell and XCell II™ Blot Module Kit CE Mark.

Protein samples were then transferred onto an Immobilon polyvinylidene difluoride membrane during 60min at 12V. For blocking purposes, membrane incubation in TBS-T 5% non fat milk was carried out 1h at room temperature with the exception of memebranes for the detection of Phospho-STAT6 which were blocked in TBS 0.05% Tween 5% BSA.

Primary antibodies were incubated in different conditions, as follows:

- anti-β Actin antibody was incubated in TBS 0.05% Tween 5% non fat milk during 1h at roomtemperature;

- anti-IL13RA1 antibody was incubated over night at 4ºC in TBS 0.1% Tween 5% non fatmilk 0,1M NaCl;

- anti-STAT-6 antibody was incubated over night in TBS 0.05% Tween 5% BSA at 4°C

- anti-Phospho-STAT6 antibody was incubated over night in TBS 0.05% Tween 5% BSA at 4°C

After washing the excess of primary antibody 3 times in TBS-0.05% Tween (or TBS-T 0.1% Tween in the case of anti-IL13Rα1), membranes were incubated 1h at room temperature with secondary antibody HRP conjugated anti rabbit (#P0448, DAKO) in TBS-0.05% Tween 5% non fat milk (or TBS-0.05% Tween 5%BSA in the case of detection of Phospho-STAT6). The excess of

secondary antibody was washed three times with TBS-0.05% Tween and protein detection was performed using the ECL (Pierce).

2.18 Statistical analysis

Statistical analysis was performed with and GraphPad Prism, Prism 5 version 5.00 for Windows software.

3 Hypothesis and aims

Hypothesis: MicroRNA-31 and microRNA-155 regulate the IL-13 pathway in Ulcerative Colitis by directly targeting IL-13Rα1 and modulating IL-13 downstream target genes.

3.1 Aims

1. To demonstrate that mRNA and protein products of IL-13 dependent genes in patients with UC are differentially expressed in paired inactive and active disease as compared to normal.

    -qPCR to assess expression of IL-13 dependent mRNA in samples from patients with active and inactive UC as compared to normal

    -Western Blotting to assess protein expression of IL13Rα1

2. To show that microRNAs predicted to target IL13Rα1 are differentially expressed in colonic samples of patients with inactive and active UC as compared to normal.

    -qPCR to assess differential expression of microRNAs predicted to target IL13Rα1 in colonic samples of patients with inactive and active UC as compared to normal

3. To demonstrate that mRNA of IL-13 dependent genes in epithelial cells isolated from patients with UC are differentially expressed in inactive and active disease as compared to normal.

    -qPCR to assess expression of IL-13 dependent mRNA expression in epithelial cells isolated from patients with UC with active and inactive UC as compared to epithelial cells isolated from normal colonic biopsies

4. To show that microRNAs miR-31 and miR-155 are differentially expressed in epithelial cells isolated from colonic samples of patients with inactive and active UC as compared to normal.

    -qPCR to assess differential expression of microRNAs miR-31 and miR-155 in epithelial cells isolated from colonic samples of patients with inactive and active UC as compared to epithelial cells isolated from normal colonic biopsies

5. To demonstrate that miR-31 directly targets IL13Rα1

    -Dual Luciferase assay to determine direct targeting of miR-31 of IL13Rα1 in HeLa cells

6. To demonstrate that miR-31 and miR-155 can modulate the expression of IL13Rα1, SOCS1 and CCL26 and mitigate the effect of IL-13 stimulation in an *in-vitro* colonic epithelial cell model (HT-29 cells)

    -qPCR to assess effects of transfection of pre-miR-31 and pre-miR-155 and their combination on the mRNA expression of IL13Rα1, SOCS1 and CCL26 in HT-29 cells

    -Western Blot to assess the differential protein expression of IL13Rα1 and STAT6 in HT-29 cells after transfection of pre-miR-31 and pre-miR-155 and their combination

7. To show that miR-31 and miR-155 can modulate the expression of IL13Rα1, SOCS1 and CCL26 and mitigate the effect of IL-13 stimulation in an *in-vitro* macrophage model (THP-1 cells)

    -qPCR to assess effects of transfection of pre-miR-31 and pre-miR-155 and their combination on the mRNA expression of IL13Rα1, SOCS1 and CCL26 in THP-1 cells

8. To demonstrate that miR-31 and miR-155 can modulate the expression of IL13Rα1, SOCS1 and CCL18 and mitigate the effect of IL-13 stimulation in an *in-vitro* model of monocyte derived human macrophages

    -qPCR to assess effects of transfection of pre-miR-31 and pre-miR-155 and their combination on the mRNA expression of IL13Rα1, SOCS1 and CCL18 in monocyte derived human macrophages

9. To demonstrate that miR-31 and miR-155 decrease expression of IL-13 dependent genes by targeting IL-13Rα1 in explant cultures of active UC compared to normal colonic tissue *ex vivo*

    -qPCR to assess effects of transfection of pre-miR-31 and pre-miR-155 and anti-miR-31 and anti-miR-155 and their combination on the mRNA expression of IL-13 dependent genes in biopsy samples from patients with inactive and active UC and normal colonic tissue.

10. To analyse the microRNA and mRNA expression profile of samples from UC patients

    - qPCR to assess microRNA and mRNA levels in samples from UC patients with active or inactive disease

11. To assess the effect of medication on the microRNA and mRNA expression profile of UC patients
    - qPCR on samples of UC patients treated with 5-ASA or Thiopurines to assess mRNA and microRNA expression
    -
12. To analyse the microRNA and mRNA expression profile of samples from CD patients

    - qPCR to assess microRNA and mRNA levels in samples from CD patients with active or inactive disease

13. To assess the effect of medication in the microRNA and mRNA expression profile of CD patients
    - qPCR on samples of UC patients treated with Thiopurines or anti-TNF-α therapy to assess mRNA and microRNA expression

4 Results 1: mRNA and microRNA profiling in unaffected inactive and active UC paired samples

4.1 Differential mRNA expression of IL-13 dependent genes in unaffected inactive and active paired sigmoid biopsies in UC as compared to normal colonic tissue

4.1.1 Introduction

IL-13 is up-regulated in UC and alongside IL-4 induces phosphorylation and activation of STAT6 via IL13Rα1 (Fuss, Heller et al. 2004). STAT-mediated signal transduction plays an important role in IBD inflammatory processes, which are characterized by a mucosal cytokine imbalance (Bouma and Strober 2003, Mudter, Weigmann et al. 2005).

UC is associated with increased colonic epithelial STAT6 phosphorylation and STAT6 inhibition with STAT6 siRNA prevents IL-13-induced apoptosis and barrier disruption. SAHA, an HDAC-inhibitor, inhibited IL-13-induced STAT6 phosphorylation, claudin-2 expression and apoptosis, and mitigated IL-13-induced reductions in trans-epithelial resistance in HT-29 cells, which makes STAT6 a possible therapeutic target in UC (Rosen, Frey et al. 2011).

Expression of IL-13 dependent genes activated through IL13Rα1 such as CCL18, SOCS1, CD23 DC-SIGN and Serpine (plasminogen activator inhibitor (PAI)-1) has been shown to be able to be influenced by miR-155 modulation in human macrophages (Martinez-Nunez, Louafi et al. 2011).

CCL18 is known to be up-regulated in various tissues in an inflammatory environment, such as skin, lungs and lymphatic tissue. It might induce expression of chemokines to attract lymphocytes, immature DC and monocytes toward various sites where macrophages and DC participate in Th1- or Th2-mediated immune responses (Strulovici-Barel, Omberg et al. 2010). CCL18 expression is regulated by the Th2 cytokines IL-4 and IL-13 and IL-10 (Meau-Petit, Tasseau et al. 2010). CCL18 expression in UC has not been reported yet.

SOCS1 is an inhibitor of STAT6 via stimulation of IL13Rα1 through IL-4 and IL-13 (Dickensheets, Venkataraman et al. 1999). Type 1 interferons have been shown to influence STAT6 expression

via SOCS1 by down-regulation of IL-13 and IL-4 in macrophages (Dickensheets, Venkataraman et al. 1999). In a clinical setting Interferon-β-1a has been shown to induce a clinical response and remission in a large subset of patients with UC by inhibition of IL-13 production facilitated by SOCS proteins (Mannon, Hornung et al. 2011). This highlights the potential role of SOCS1 in UC.

SERPINE1 has been shown to be a crucial player in wound healing and high levels of Serpine blocks smooth muscle migration and therefore impairs wound healing (Holubar and Harvey-Banchik 2007). Serpine has been shown to be up-regulated by IL-13 (Martinez-Nunez, Louafi et al. 2011).

MMP-9 is induced by IL-13 stimulation (Deaton, Forsyth et al. 1951) and over expression of MMP9 in active IBD causes the destruction of tissue (Harvey and Sloviter 2005, Harvey, Bothma et al. 2005). A sevenfold increase of MMP-9 has been observed in inflamed IBD tissue compared to controls (Postlethwait and Bradshaw 1950).

Despite UC mainly being a Th2-like response in which IL-13 (and IL-5) plays a pivotal role (Strober and Fuss 2011), increased TNF-α mRNA gene expression in inflamed colorectal mucosa in UC has been shown by qPCR and was confirmed by immunohistochemistry (Harvey, Naciti et al. 2004). ACT 1 and ACT 2 studies (Infliximab), as well as the CHARM trial (Adalimumab) demonstrated a beneficial effect of Anti-TNF therapy in UC (Rutgeerts, Sandborn et al. 2005, Colombel, Sandborn et al. 2007) confirming the importance of TNF-α in the pathogenesis of UC.

Eotaxin-3 (CCL26) is a Th2 driven cytokine whose expression is stimulated by IL-13 through the IL13Rα1. CCL11/eotaxin-1, CCL24/eotaxin-2 and CCL26/eotaxin-3 are known to attract CCR3-expressing Th2-polarized lymphocytes. A recent study revealed raised CCL26 levels in blood and colonic biopsies of patients with UC as compared to normal controls (Manousou, Kolios et al. 2010).

IL13Rα1 expression was measured to detect whether there is a difference in its expression in normal, unaffected inactive and active UC. Increased expression of IL13Rα1 and IL-13Rα2 were previously reported in patients with UC (Mandal and Levine 2010), but these were compared to normal tissue and not conducted in matched paired samples in contrast to this study of patients with distal UC. The advantage of studying paired unaffected un-inflamed and inflamed biopsy

samples from the same patients that it provides the perfect control when looking at gene expression due solely to inflammation and eliminates possible genetic differences in the basal expression of various genes.

In this study we compared normal tissue from eleven patients who attended for polyp follow up from pinch biopsies taken from unaffected sigmoid colon to samples from sigmoid colon of 11 patients suffering from UC affecting the distal colon. Biopsies were taken from the unaffected, inactive, macroscopically normal mucosa (endoscopic Mayo score 0-1) within 10 cm of the active disease (endoscopic Mayo score 3-4). Biopsies were frozen in cryovials in liquid nitrogen and stored at -80C.
RNA was isolated (see 3.15), then reverse transcribed and assayed by qPCR (see 3.16) to detect the mRNA expression levels of CCL18, SOCS1, CCL26, Serpine, MMP-9, TNF-α and IL13Rα1.

Data was analysed with GraphPad Prism, Prism 5 version 5.00 for Windows software. Data was compared to normal, but also an analysis of the paired UC samples from each patient was evaluated.

4.1.2 mRNA expression of IL-13 dependent genes in unaffected inactive and active tissue biopsies in UC as compared to normal colonic tissue

Major differences were found in the expression of mRNA of IL-13 dependent genes and the expression of IL13Rα1 in the 3 groups of normal colonic biopsies, unaffected inactive and active UC. Demographic data of the patients is summarized in ***Table 8***. Normal colonic biopsies, unaffected inactive and active UC biopsies were obtained as in ***section 2.7 and 2.8***, and RNA was extracted (quality of RNA was confirmed with a nanodrop aiming for a 260/280 ratio of 1.95-2.05 for all samples used) (***see 2.16***). RT-qPCR was performed as explained in ***2.17 and 2.18***. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of healthy controls.

Figure 13 shows the detailed results of the expression of mRNA of CCL18, SOCS1, SERPINE1, MMP9, TNF-α, CCL26 and IL13Rα1 in the 3 groups of patients. CCL18, SOCS1, CCL26 and MMP9 mRNA levels were significantly up-regulated in active UC as compared to unaffected, inactive areas of UC. Although only statistically significant in the SERPINE1 and CCL26 groups, a clear up-regulation was also observed in these IL-13 dependent genes in active UC compared to normal samples from the control population. No significant difference was found in the expression of TNF-α in all the groups. IL13Rα1 was observed to be significantly down-regulated in active UC as compared to unaffected, inactive UC and normal colon.

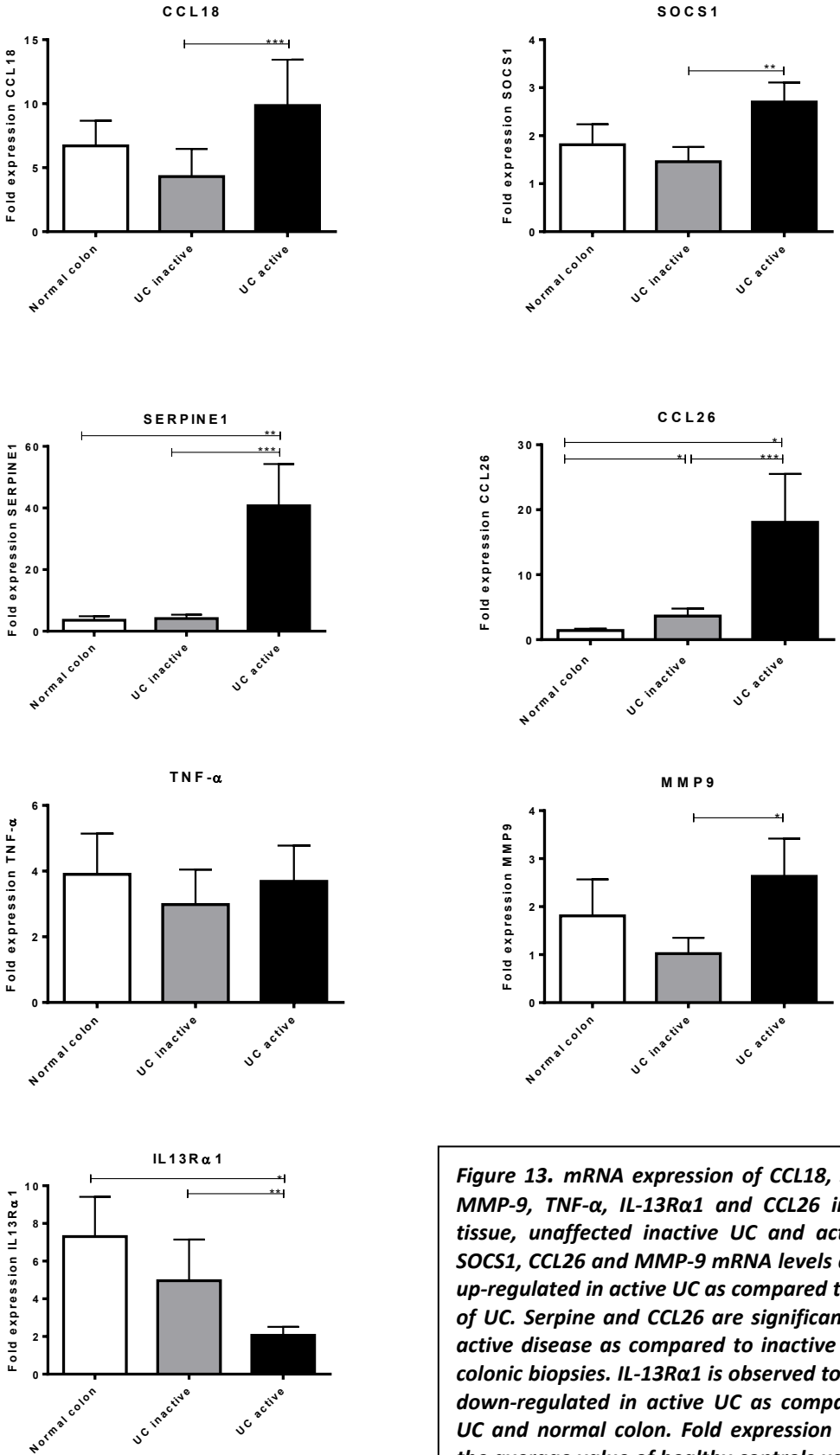


Figure 13. mRNA expression of CCL18, SOCS1, Serpine, MMP-9, TNF-α, IL-13Rα1 and CCL26 in normal colon tissue, unaffected inactive UC and active UC. CCL18, SOCS1, CCL26 and MMP-9 mRNA levels are significantly up-regulated in active UC as compared to inactive areas of UC. Serpine and CCL26 are significantly increased in active disease as compared to inactive UC and normal colonic biopsies. IL-13Rα1 is observed to be significantly down-regulated in active UC as compared to inactive UC and normal colon. Fold expression is calculated to the average value of healthy controls using GAPDH as a normaliser.*: P-value<0.05, **: P-value<0.01, ***: P-

4.1.3 Protein expression of IL13Rα1 in normal colon, unaffected, inactive UC and active UC

Significant down-regulation of IL13Rα1 mRNA was observed in paired active UC biopsies as compared to normal controls and unaffected inactive UC.

We hypothesise that high levels of miR-31 and miR-155 in active UC are targeting protein translation of the IL13Rα1 and therefore down-regulate IL13Rα1 to protect the tissue from excessive stimulation of the IL-13/STAT6 pathway.

Western blotting was conducted on biopsy samples from 6 patients with unaffected inactive UC, 6 patients with active UC and 6 normal controls.

This data shows that IL13Rα1 protein expression was significantly reduced in human colonic biopsies in active UC as compared to normal colonic tissue and unaffected, inactive UC tissue. ***Figure 14*** shows protein expression in Western Blots of 6 normal subjects, 6 patients with unaffected inactive and 6 patients with active UC. Patients with active ulcerative colitis had severe inflammation with a Mayo score greater than 2. Unaffected, inactive samples were macroscopically normal (Mayo score 0), indicating deep remission with complete mucosal healing. ***Figure 15*** shows the densitometry of the western blots in ***Figure 14***. According to manufacturers notes Anti-IL-13Rα1 (sc-25849, Santa Cruz Biotechnology Inc., Heidelberg, Germany) represents the lower of the double band at about 50kD (***Figure 14)***.

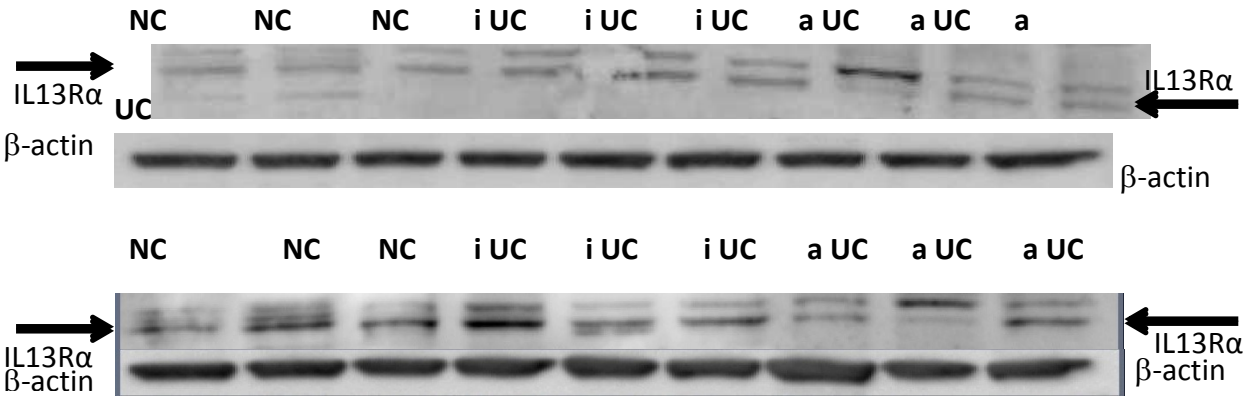


Figure 14. Representative Western Blot of colonic biopsies of normal controls (NC), patients with unaffected inactive UC (i UC) and active UC (a UC). N=6 for each group. According to manufacturers notes Anti-IL-13Rα1 (sc-25849, Santa Cruz Biotechnology Inc., Heidelberg, Germany) represents the lower of the double band at about 50kD.

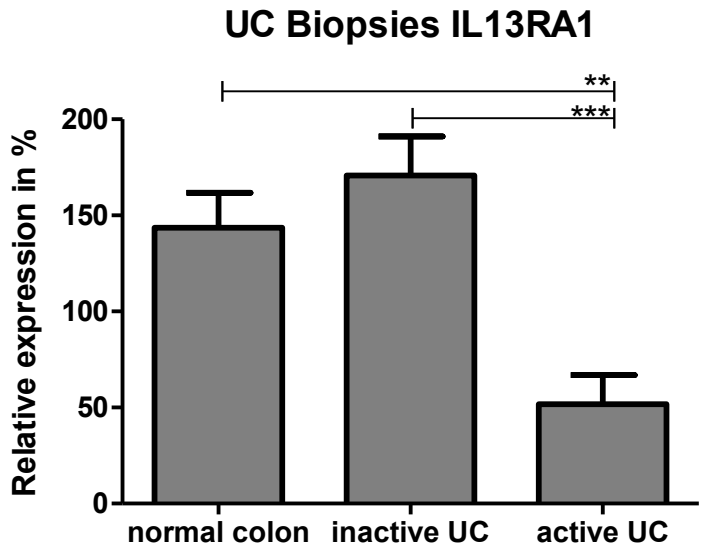


Figure 15. Densitometry of Western blots in samples from normal controls), unaffected inactive UC and active UC (N=6 in each group). IL13Rα1 protein expression is significantly down-regulated in active UC samples as compared to control samples and inactive UC. Relative expression is calculated to the average value of normal controls in percent.

Results confirm findings from mRNA data in paired unaffected inactive and active UC compared to normal controls showing a significant down-regulation of IL13Rα1 in protein expression in inflamed UC biopsies compared to unaffected, inactive UC and normal controls (***Figures 14*** and ***15***)

4.2 Expression of microRNAs targeting IL13Rα1 in normal, unaffected, inactive and active sigmoid biopsies in UC

4.2.1 Introduction

IL-13 signalling through IL13Rα1 activating STAT6 plays an important role in the pathogenesis of UC. This has been highlighted by studies showing that ultimate down-regulation of the JAK-STAT6 pathway with siRNAs or HDAC inhibitors leads to a reduction of the toxic effects of IL-13 to epithelial cells (Rosen, Frey et al. 2011). Interferon-β-1a has been used to down-regulate STAT6 via SOCS1 activation, another IL-13 regulated gene to achieve clinical remission in a subset of patients with active UC (Mannon, Hornung et al. 2011). MicroRNAs targeting IL13Rα1 might well be able to reduce IL-13 induced JAK-STAT6 activation via down-regulation of IL13Rα1 and therefore influence the severity of inflammation, similarly to studies describing down-regulation of STAT6 via siRNA, HDAC inhibitors and Interferon-β-1a (Rosen, Frey et al. 2011) (Mannon, Hornung et al. 2011).

This study demonstrated IL13Rα1 mRNA and protein expression was significantly decreased in active UC compared to normal and unaffected inactive UC tissue. We were interested in exploring if microRNAs which potentially targeted IL13R α1 were differentially expressed in these tissues and therefore could provide a mechanism for these changes in mRNA levels and protein expression (***Figures 13, 14*** and ***15***). Using several different bioinformatics software packages, (TargetScan: www.TargetScan.org, microrna.org (http://www.microrna.org/microrna/home.do) and PITA algorithm (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) we have identified a number of microRNAs predicted to target IL13Rα1 directly (see ***section 2.3***).

Fasseu *et al.* investigated differential expression of microRNAs in IBD in inflamed and inactive samples compared to normal colonic biopsies. A number of microRNAs targeting IL13Rα1 were found to be up or down-regulated were highlighted in this paper. MiR-31 was shown to be elevated in inflamed UC and also dysregulated in CD and miR-196 was down-regulated in UC. MiR-148/152, miR-183 and miR-155 were increased in CD, and miR-324 was elevated in UC and CD (Fasseu, Treton et al. 2010). MiR-155 has been shown to be up-regulated in active UC by

another study (Takagi, Naito et al. 2010). No reports of dysregulation of miR-27ab or miR-374 in IBD have been reported in the literature.

In this study we have measured the expression of 8 candidate microRNAs (miR-27ab, miR-31, miR-148/152, miR-155, miR-183, miR-196ab, miR-324-5p and miR-374/374ab) predicted to directly target IL13Rα1 (see 3.3). MiR-324-5p and miR-155 were also identified as potentially targeting SOCS1 and miR-155 has been shown to target SOCS1 (Wang, Hou et al. 2010). RNA extraction and RT-qPCR were performed as explained in sections ***2.16, 2.17 and 2.18***. RNU44 was used as a normalizer gene.

4.2.2 microRNAs predicted to target IL13Rα1 are differentially expressed in normal colonic biopsies, unaffected inactive and active UC

Figure 16 shows the differential expression of microRNAs predicted to target IL13Rα1 in macroscopically normal biopsies from unaffected, inactive sigmoid colon (endoscopic Mayo score 0-1) and active (endoscopic Mayo score 2-3) paired samples from sigmoid colon of patients with UC (N=11), compared to sigmoid biopsies of normal healthy controls. Paired samples were taken from inflamed areas (distal colitis with Mayo score > 2) and inactive areas of the sigmoid colon (Mayo score 0-1) above the active segment in 11 individual patients with active distal UC.

The results (see ***Figure 16***) revealed highly significant up-regulation of miR-31 in active UC compared to normal samples. Comparison of the paired unaffected inactive and active UC samples revealed a significant up-regulation of miR-31 using the Wilcoxon matched pairs test (***Figure 16***).

Highly significant results were also seen in the expression of miR-155 comparing normal to active UC (***Figure 16***) as well as paired unaffected inactive and active samples using the Wilcoxon matched pairs test (***Figure 16***).Significant change was also observed with up-regulation in unaffected inactive UC compared to normal tissue, and a non-significant down-regulation in active disease compared to unaffected inactive disease (***Figure 16***).

Non-significant up-regulation in the expression of miR-27a, miR-152, miR-183, miR-196 or miR-374 in active disease as compared to normal and unaffected inactive disease was observed. MiR-196 and miR-324 were down-regulated in active disease as compared to unaffected, inactive disease (***Figure 16***).

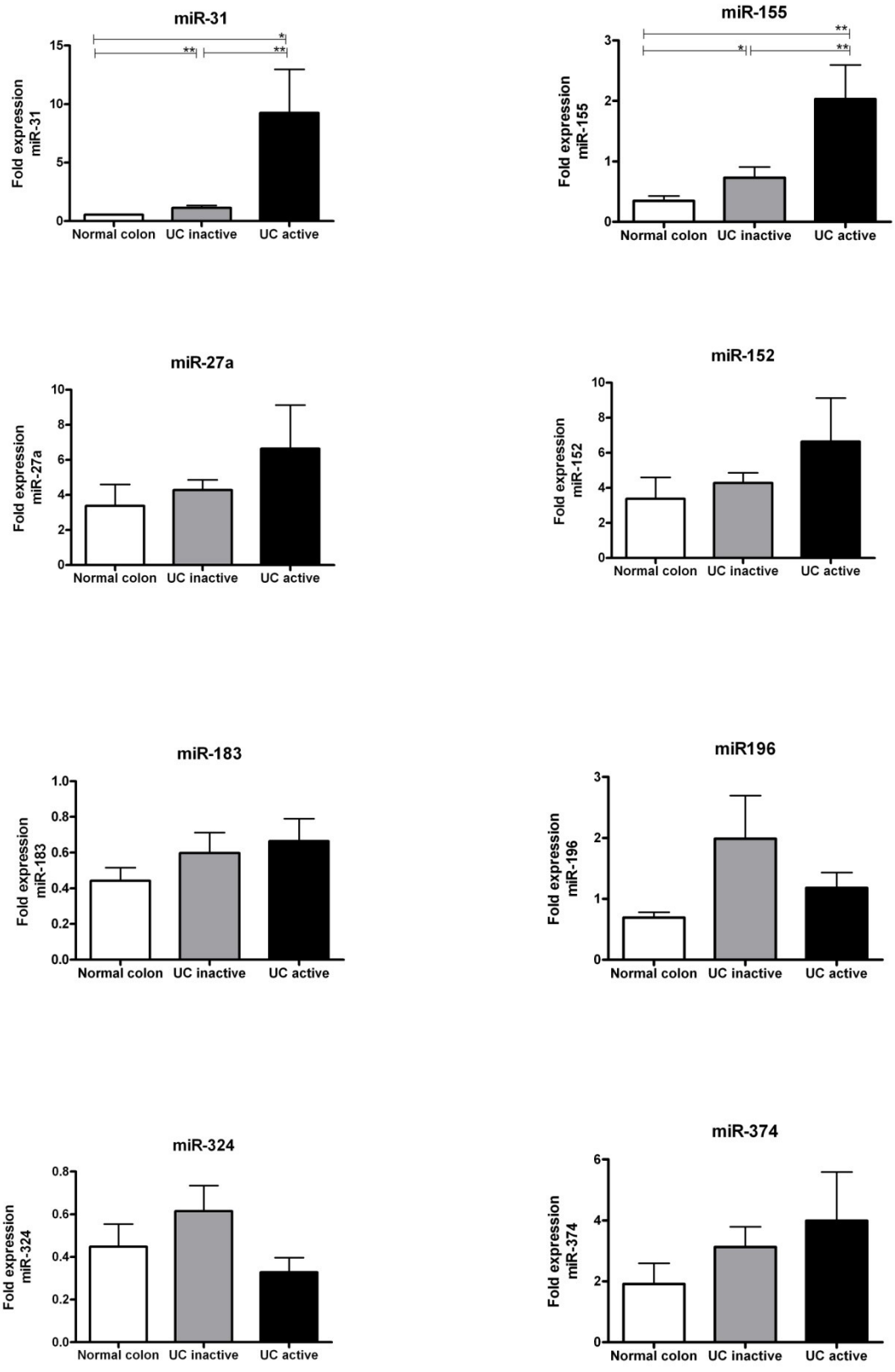


Figure 16. MicroRNA profiling of normal colon versus active and unaffected, inactive UC samples. Expression of miR-27ab, miR-31, miR-152, miR-155, miR-183, miR-196ab, miR-324-5p and miR-374/374ab were measured. RNU44 was used as a normalizer gene. Fold expression was calculated to the average value of healthy controls.*: P-value<0.05, **: P-value<0.01, ***: P-value <0.001. N=11 in each group.

4.3 Expression of microRNAs and IL-13 dependent genes in epithelial cells from sigmoid UC biopsies

4.3.1 Introduction

In the previous chapter we were able to show differential expression of mRNA and microRNAs in biopsy samples, containing the epithelial layer and the submucosa (see ***sections 2.7, 2.8, 2.16, 2.17*** and ***2.18***), from patients with unaffected inactive and active UC. In particular we were interested how this change was affecting epithelial cells, as there is good evidence that the breach of the epithelial barrier in active UC is one of the major factors contributing to the pathophysiology of UC. To investigate as to whether there were differences in the mechanism of the IL-13 and NK-T cells mediated inflammation in epithelial cells as compared to the submucosa, comprising macrophages and dendritic cells of the innate immune system and the components of the adaptive immune system, we isolated epithelial cells from normal controls, unaffected, inactive UC and active UC (for isolation protocol see ***section 2.14***). Demographic data for the eplithelial cell isolation is shown in ***Table 9***.

As previously described, UC is driven by a Th2-like response whose major factor is NKT cells producing IL-13 (and IL-5) (Strober and Fuss 2011). It was established that NKT cells as well as IL-13 have a negative effect on epithelial cells leading to increased mucosal permeability (Heller, Fuss et al. 2002). This was confirmed by a study of Heller *et al.* showing that IL-13 decreases the mucosal resistance in HT-29 cells and also increases the production of claudin-2, leading to increased epithelial barrier permeability in UC (Heller, Florian et al. 2005).

Criteria for selection of biopsies from patients with UC consisted of applying the MAYO Endoscopic sore for unaffected inactive (Mayo score 0-1) and active samples (Mayo score 2-3), and these two groups were paired with age matched normal controls (N=5 in each group).

4.3.2 mRNA expression of IL-13 dependent genes and IL13Rα1 in epithelial cells from normal controls, unaffected, inactive UC and active UC

Expression of SOCS1, CCL18, SERPINE1, MMP9, TNF-α and IL13Rα1 was measured in epithelial cells of normal colonic epithelial cells from healthy patients and epithelial cells from patients with unaffected inactive and active UC. CCL18, SERPINE1, MMP9 and TNF-α were not sufficiently expressed in normal and unaffected inactive UC to allow a comparison. CCL18 expression in inflamed UC was thought to be a contamination of lamina propria mononuclear cells such as macrophages and dentritic cells, as CCL18 was undetected in normal and un-inflamed samples and was also not expressed in HT-29 cells, an *in vitro* epithelial cell line used for functional studies in this project.

Figure 17 shows the expression of IL13Rα1 and the IL-13 dependent gene SOCS1 in epithelial cells of normal controls, unaffected inactive and active UC isolated from biopsy samples. IL13Rα1 is significantly down-regulated in inflamed epithelial cells isolated from UC patient biopsy samples compared to normal controls and unaffected inactive UC epithelial cell isolates. Expression of SOCS1 in the same samples is significantly increased in unaffected inactive and active UC, similar to the expression in whole biopsy samples. SOCS1 is also significantly increased when unaffected inactive and active samples are compared, reflecting the increased IL-13 stimulation in active UC. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of healthy controls.

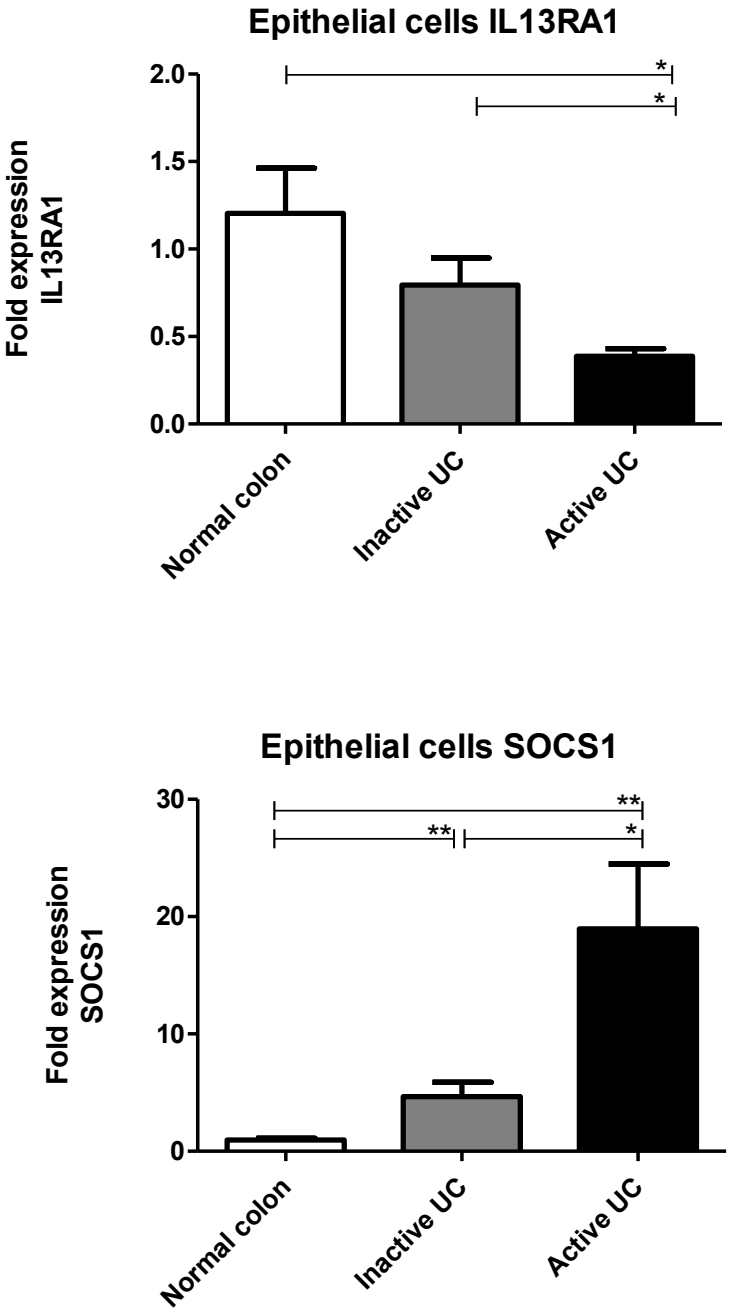


Figure 17. Expression of IL13Rα1 and the IL-13 dependent gene SOCS1 in epithelial cells of normal controls, unaffected, inactive and active UC isolated from biopsy samples. Significant down-regulation of IL13 Rα1 in unaffected inactive and active UC epithelial cell is demonstrated as compared to normal samples. SOCS1 is significantly up-regulated in unaffected inactive and active samples. SOCS1 is also significantly increased in the active sample as compared to the unaffected inactive sample. N=5 in each group. **GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of healthy controls.** ****: P-value<0.05, **: P-value<0.01, ***: P-value <0.001. N=11 in each group.***

4.3.3 microRNA expression of miR-31 and miR-155 in epithelial cells from normal controls, unaffected, inactive and active UC

4.3.3.1 Introduction

MiR-155 was found to be up-regulated in IBD tissue (Fasseu, Treton et al. 2010), especially in UC (Takagi, Naito et al. 2010). MiR-31 was also demonstrated to be up-regulated in UC (Fasseu, Treton et al. 2010).

These findings were confirmed with data from this series in human UC biopsy samples, showing both microRNAs significantly up-regulated in inflamed paired samples as compared to unaffected inactive UC biopsies (***Figure 16***). IL13Rα1 in active UC biopsy samples was demonstrated to be significantly down-regulated as compared to normal and unaffected inactive UC biopsies from normal sigmoid biopsies (***Figures 13, 14 and 15***).

MiR-155 directly targets IL13Rα1 (Martinez-Nunez, Louafi et al., 2011) as shown by our group , but also targets SOCS1 (Wang, Hou et al. 2010) and miR-31 was predicted to directly target IL13Rα1 by microRNA bioinformatics tools such as TargetScan (see ***section 2.3*** and ***Figures 6 and 7***).

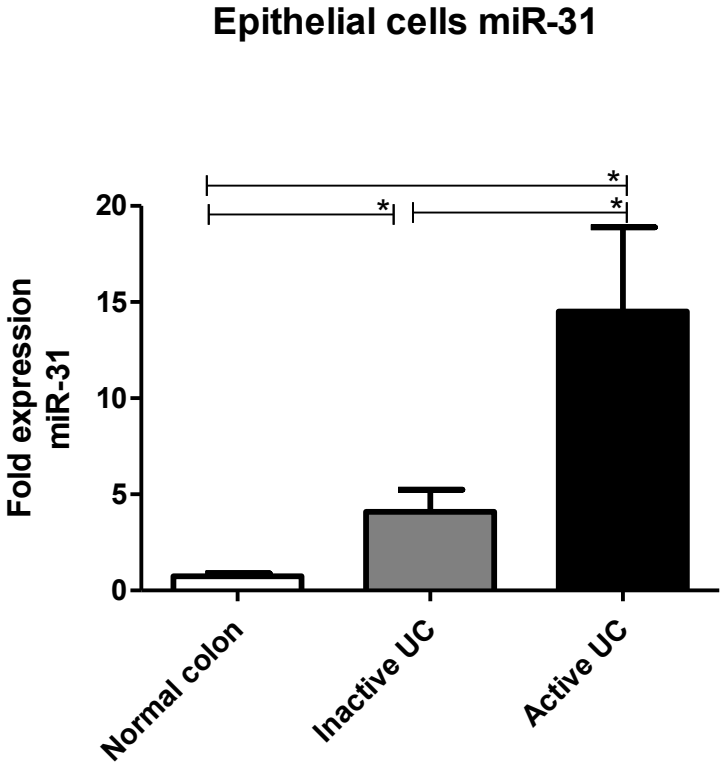
As a result of these findings of a significantly down-regulated IL13Rα1 in active UC biopsies, we were interested if the two microRNAs targeting IL13Rα1, miR-31 (predicted) and miR-155, were also significantly up-regulated, re-affirming the hypothesis of the protective nature of these microRNAs trying to mitigate the IL-13/STAT6 pathway activation in order to reduce the inflammatory response.

4.3.3.2 microRNA expression of miR-31 and miR-155 in epithelial cells from normal controls, unaffected inactive and active UC

Figure 18 demonstrates the expression of miR-31 and miR-155 in epithelial cells isolated from normal control biopsies and patients with unaffected inactive and active UC. RNU44 was used as a normalizer gene. Fold expression was calculated to the average value of healthy controls.

MiR-31 is significantly up-regulated in unaffected inactive (5 fold) and active (15 fold) epithelial cells from patients with UC as compared to normal epithelial cells (***Figure 18***).

MiR-155 is elevated 1.5 fold in unaffected inactive epithelial cells from UC patients and nearly 5 fold (significant) in active epithelial cells from UC patients as compared to epithelial cells from normal control samples (***Figure 18***).



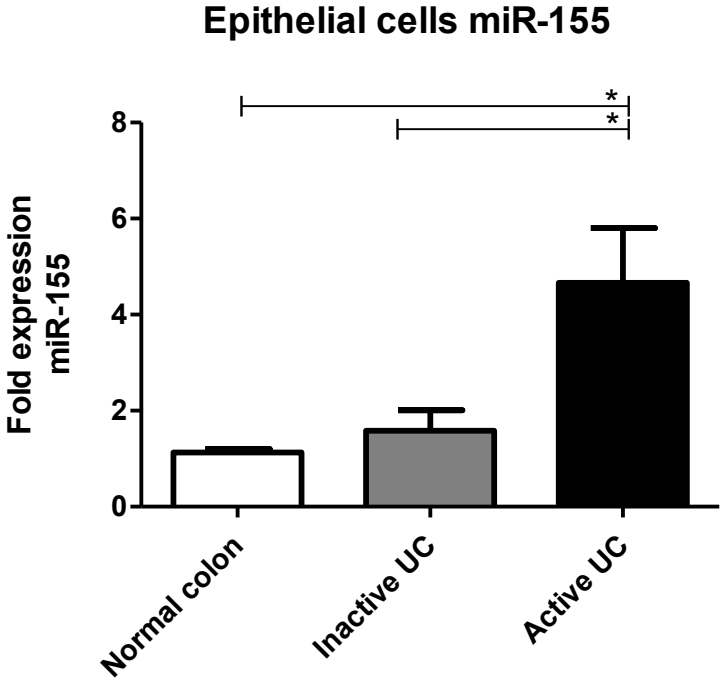


Figure 18. MiR-31 and miR-155expression in epithelial cells from normal colon, unaffected inactive and active UC. Both miR-31 and miR-155 were significantly up-regulated in epithelial cells from active UC patients compared to normal epithelial cells. N=5 in each group. RNU44 was used as a normalizer gene. Fold expression was calculated to the average value of healthy controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

4.4 Discussion

UC is characterized by the over expression of IL-13 alongside IL-4 inducing IL-13-dependent activation of STAT6 via IL13Rα1 (Fuss, Heller et al. 2004). Increased STAT6 phosphorylation has been associated with UC and its inhibition with siRNAs or HDAC inhibitors has been shown to reduce the toxic effects of IL-13 on epithelial cells (Rosen, Frey et al. 2011). IL13Rα1 expression was reported to be differential in normal, unaffected inactive and active UC. Increased expression of IL13Rα1 and IL-13Rα2 was previously found in patients with UC (Mandal and Levine 2010), but this was not comparing active and unaffected inactive disease of the matched paired patient samples. SOCS1 also has been shown to influence the level of inflammation by down-regulating STAT6, after induced by Interferon-β-1a in patients with UC (Mannon, Hornung et al. 2011).

CCL18 has been described in UC in this study for the first time. It is known to induce expression of chemokines attracting lymphocytes, dendritic cells and monocytes to the site of inflammation (Schutyser, Richmond et al. 2005) and has been shown to be up-regulated in other inflammatory conditions. Being regulated by Th2 cytokines IL-4 and IL-13 and IL-10 (Meau-Petit, Tasseau et al. 2010), it was unsurprising that it was raised in active UC. Compared to normal colonic biopsies the expression of CCL18 mRNA was found twofold increased in active UC, and interestingly the level in unaffected inactive UC was slightly lower than the basal level in normal samples (***Figure 13***). The statistically significant rise between the paired unaffected inactive and active UC samples (***Figure 13***) is remarkable considering their close proximity to the inflamed mucosa (about 10cm) in our patient population. Overall this novel finding of increased CCL18 expression in active UC could be a good marker of activity in UC and may help to determine phenotype and extent of disease.

SOCS1, another IL-13 induced gene was significantly raised in active UC compared to unaffected inactive UC (***Figure 13***). SOCS1, being an inhibitor of STAT6 signalling, has been shown to exert anti-inflammatory effects by down-regulating STAT6 in UC in the context of Interferon-β-1a therapy in patients with UC (Mannon, Hornung et al. 2011). High SOCS1 levels may therefore be protective in UC and would be desirable in unaffected segments of UC. Our data shows a slight decrease of SOCS1 in unaffected inactive UC as compared to normal tissue, but a significant increase in active disease (***Figure 13***). The increase in active disease might be an attempt to

decrease STAT6 activation and therefore decrease the inflammatory response. Interestingly, SOCS1 levels have been shown to be increased by anti-miR-155 in human macrophages after stimulation with IL-13 (Martinez-Nunez, Louafi et al. 2011) and have been shown to be a direct target of miR-155 (Wang, Hou et al. 2010). Together, these observations and our results make targeting the IL13Rα1 with microRNAs an interesting possibility to modulate the expression of SOCS1 in UC serving as a possible therapeutic target.

SERPINE1, regulated by IL-13, has been shown to be an important player in wound healing with high levels of SERPINE1 shown to block smooth muscle migration impairing tissue repair (Holubar and Harvey-Banchik 2007). SERPINE1 was significantly up-regulated in active disease compared to unaffected inactive UC samples and normal biopsies (***Figure 13***) in this series and may reflect the increased stimulation of IL-13 via IL13Rα1. These results suggest that it may be a good marker of activity, although it has not been well described in the literature in UC yet. Our results (***Figure 13***) are in agreement with the well documente increased expression of MMP9 (Harvey and Sloviter 2005, Harvey, Bothma et al. 2005) which affects the degradation of the ECM.

Despite the fact that the main detrimental cytokine in UC is IL-13 produced by NKT cells, TNF-α expression has been shown to be up-regulated in UC and correlates to the grade of inflammation in untreated patients (Olsen, Goll et al. 2007). In this study mRNA expression of TNF-α was not significantly different in all the groups (***Figure 13***) which may well reflect the fact that most of patients have been on various treatments for UC. TNF-α may play a more dominant role in the pathogenesis of UC in a subset of patients, as clinical trials with anti-TNF-α compounds show a long-term remission rate of about a third of patients with Infliximab (Rutgeerts, Sandborn et al. 2005) and about 1 in 5 patients treated with Adalimumab achieved remission at 8 weeks (Reinisch, Sandborn et al. 2011).

CCL26 was significantly increased in active disease as compared to normal controls (***Figure 13***) and this is in keeping with previously published work defining its role in the IL-13/STAT6 pathway and its increased expression in active UC (Blanchard, Durual et al. 2005, Manousou, Kolios et al. 2010).

IL13Rα1 has been previously reported to be up-regulated in UC (Mandal and Levine 2010). Our data suggest a significant reduction of IL13Rα1 mRNA in active UC as compared to unaffected, inactive UC and normal control samples. A significant down-regulation of IL13Rα1 mRNA in our paired biopsy samples was confirmed by significantly decreased IL13Rα1 protein expression in colonic biopsies from patients with active UC (endoscopic Mayo score 2-3) as compared to normal controls and unaffected inactive UC (***Figures 14*** and ***15***).

The increase of IL13Rα1 in UC reported by Mandal *et al* was demonstrated in a subset of samples of epithelial cells from patients with UC who underwent resection. No data regarding the level of inflammation of the specimens or the circumstances of resection are given. No information is available whether these patients underwent colectomy for failing medical rescue treatment or underwent colectomy for other reasons such as dysplasia or cancer. Medical treatment might well influence the expression of IL13Rα1 in UC, addressed later in this thesis (***Chapter7***). The numbers in the study of Mandal et al were also much lower than in this study and did not take the degree of inflammation of the resection specimens into account. Furthermore the study of Mandal *et al* (Mandal and Levine 2010) was looking at epithelial cells only as our qPCR and Western blot results include full biopsies representing the mucosa and sub-mucosa.

Reduction of IL13Rα1, along with the early increase in SOCS1 might be an attempt of auto-regulation to break the pro-inflammatory cycle through IL-13 signalling via IL13Rα1 to decrease STAT6 activation.

MicroRNAs identified to target or predicted to target IL13Rα1 could therefore play a role in the course of disease influencing the activation of the JAK-STAT6 pathway and the direct toxic effect of IL-13 and NKT cells to the epithelium in UC. We therefore analysed the expression of these microRNAs in our samples of normal, unaffected inactive and active UC biopsies of the sigmoid colon. Our results clearly favoured miR-31 and miR-155 as candidates for further studies. Although all microRNAs tested (miR-27ab, miR-31, miR-148/152, miR-155, miR-183, miR-196ab, miR-324-5p and miR-374/374ab) were differentially expressed in the various conditions, miR-31 and miR-155 were significantly up-regulated in active disease as compared to normal colonic biopsies and unaffected, inactive UC (***Figure 16***).

These significant results in the differential expression of miR-155, which directly targets IL13Rα1 (Martinez-Nunez, Louafi et al., 2011) and miR-31 (predicted to target IL13Rα1, see ***2.3***. and ***Figures 6*** and ***7***) in our study population in combination with the clinical importance of miR-31 and miR-155 in UC called for closer examination of the effects of these microRNAs in the IL-13 pathway in UC.

Concomitantly with the up-regulation of miR-31 and miR-155, we found a significant down-regulation of IL13Rα1 on mRNA level in active paired UC samples as compared to normal and paired un-inflamed samples in these same samples where miR-31 and miR-155 were significantly elevated. Importantly, we showed that protein expression of IL13Rα1 was significantly reduced in inflamed UC biopsies as compared to normal controls and unaffected inactive UC samples (***Figure 16***).

Our findings confirm the results of previous studies which showed miR-31 up-regulated in UC (Fasseu, Treton et al. 2010, Olaru, Selaru et al. 2011) and define the expression in the active disease compared to the unaffected inactive areas of UC patients (***Figure 16***). In addition we were able to show that this significant change in the expression of miR-31 happens within the same patients in active UC in close proximity to the unaffected inactive segment which shows significantly lower expression of miR-31.

MiR-31 has a variety of interesting connections to IBD. Firstly, its genomic location close to a cluster of interferons (***Figure 4***) is suggestive of a possible common regulation with this cluster. Interferon-β-1a, located in that region of chromosome 9, has been shown to exhibit therapeutic effects inducing remission in a subset of patients with UC via SOCS proteins (Mannon, Hornung et al. 2011). Secondly, miR-31 has been found to have a wide variety of other predicted targets highlighting the potential importance of this microRNA in the regulation of the immune response by targeting FOXP3, or HDAC1, HDAC2 and HDAC9 (Rouas, Fayyad-Kazan et al. 2009). MiR-31 has been found to influence T-regs by suppressing the expression levels of FOXP3 (Rouas, Fayyad-Kazan et al. 2009) and the down-regulation of STAT6 activation by the HDAC inhibitor SAHA in UC (Rosen, Frey et al. 2011) is an interesting finding considering that miR-31 targets HDAC1, HDAC2 and HDAC9 (Rouas, Fayyad-Kazan et al. 2009). These findings highlight the potential benefits of modulating the expression of this microRNA in IBD.

E-selectin, which recruits neutrophils to be trans-located to a site of inflammation, is targeted by TNF-induced miR-31 and was shown to inhibit neutrophil adhesion to endothelial cells suggesting that miR-31 has a potential role in anti-inflammatory therapy (Suarez, Wang et al. 2010). Moreover, miR-31 expression has been shown to increase in a stepwise fashion as tissue goes from normal to chronically inflamed to actively inflamed to neoplastic (Olaru, Selaru et al. 2011). Thus, controlling the expression of miR-31 could have a potential role to modulate the neoplastic transformation of IBD tissue.

Taking into consideration the involvement of miR-31 in T cell regulation, gene transcription, neutrophil recruitment and oncogenesis in combination with its potential effect on regulating the IL-13 pathway by targeting IL13Rα1, miR-31 is a very interesting candidate for further studies.

MiR-155 was found to be up-regulated in IBD tissue (Fasseu, Treton et al. 2010), especially in UC (Takagi, Naito et al. 2010). This was confirmed by our results showing a significant up-regulation of miR-155 in the inflamed UC samples (***Figures 16***). MiR-155 over-expression was also expected considering the extensive involvement of miR-155 in the innate and adaptive immune system and inflammatory pathways.

MiR-155 has been shown to regulate the inflammatory response after pathogen recognition by Toll-like receptors (TLRs) on monocytes or macrophages (Bakirtzi, Hatziapostolou et al. 2011). Its expression is induced by bacterial stimulation and a number of pro-inflammatory cytokines such as IFN-γ, IFN-β or TNF (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007). Our group has previously shown that miR-155 directly targets IL13Rα1 in human macrophages (Martinez-Nunez, Louafi et al. 2011) and also its involvement in the maturation of dendritic cells regulating proteins involved in the cellular immune response against pathogens (Martinez-Nunez, Louafi et al. 2011).

Activation of NF-κB, critical to innate and adaptive immunity, cell proliferation, inflammation and tumour development, has been demonstrated to correlate with miR-155 up-regulation. This provides a possible link in the involvement of miR-155 in the switch between inflammation and cancer in IBD patients (Ma, Becker Buscaglia et al. 2011).

MiR-155 and has been shown to target SOCS1 (Wang, Hou et al. 2010) and work in our group has shown that miR-155 regulates SOCS1 in human macrophages (Martinez-Nunez, Louafi et al. 2011). MiR-155 maintains T-regs proliferation and homeostasis by the down-regulation of SOCS1 expression and it also directly targets Foxp3 in these cells contributing to their development (Rodriguez, Vigorito et al. 2007, Kohlhaas, Garden et al. 2009). Yao *et al*. has demonstrated that miR-155 enhanced Treg and Th17 cells differentiation and IL-17A production by targeting SOCS1 (Yao, Ma et al. 2012).

MiR-31 and miR-155 are therefore involved in different inflammatory pathways and regulation of innate and adaptive immunity. Moreover, these two microRNAs are implicated in inflammation to cancer progression. They could therefore be important players in the pathogenesis of inflammatory bowel disease and its neoplastic transformation, especially in UC by directly modulating the IL-13 pathway through targeting IL13Rα1. The dysregulation of these two microRNAs in inflamed UC tissue in combination with the differential expression of IL-13 dependent genes in our paired samples formed the basis of the *in vitro* experiments to determine their exact individual function and possible synergy in their effects on the IL-13 pathway in UC.

Given the important role of the epithelial barrier in IBD (see ***section 1.4.1.1***) we analysed the expression of miR-31 and miR-155 as well as the expression of IL-13-dependant genes and IL13Rα1 in epithelial cells isolated from UC samples.

Our findings of significantly elevated levels of miR-31 and miR-155 (***Figure 18***) with the corresponding down-regulation of IL13Rα1 (***Figure 17***) in inflamed UC samples of epithelial cell isolates as compared to normal controls corroborated with our data from unaffected inactive and active paired biopsies in UC (***Figures 13, 14 and 15***). Importantly, microRNA expression of miR-31 and miR-155 in epithelial cells of normal colon and UC has previously not been reported in the literature.

Our results in epithelial cells as well as biopsies are in line with published literature in biopsy samples reporting miR-155 up-regulated in IBD tissue (Fasseu, Treton et al. 2010), especially in UC (Takagi, Naito et al. 2010) and miR-31 was also demonstrated to be increased in UC (Fasseu, Treton et al. 2010).

CCL18, SERPINE11, MMP9 and TNF-α mRNA were not sufficiently expressed in normal and unaffected inactive UC epithelial cell isolates to allow a comparison with active UC epithelial cell isolates. CCL18 expression in inflamed UC was thought to be a contamination of lamina propria mononuclear cells such as macrophages and dentritic cells, as CCL18 was undetected in normal controls and un-inflamed UC colonic epithelial cell isolates and was also not expressed in HT-29 cells, an *in vitro* epithelial cell line we used for functional studies in this project (see ***section 5.2***).

Generally, the epithelial cells were isolated from 5 pinch biopsies of the same area in the sigmoid colon and the fact that some genes (SERPINE11, MMP9 and TNF-α) were not found to be significantly expressed in normal and un-inflamed samples, probably just reflects the small amount of RNA isolated and reverse transcribed leading to amplification below the necessary threshold to be detected in the PCR, rather than these genes not being expressed in normal and un-inflamed epithelial cells.

Knowing that both miR-31 and miR-155 (directly targeting IL13Rα1) are up-regulated in inflamed UC epithelial cells with a corresponding down-regulation of IL13Rα1 in inflamed UC epithelial cells sets the scene to test our hypothesis with functional *in vitro* experiments with a colonic epithelial cell line to investigate the observations we made in patient samples.

5 Results 2: Functional Role of miR-31 and miR-155 in UC models

5.1 MiR-31 directly targets the IL13Rα1 3'UTR

5.1.1 Introduction

MiR-31, predicted to target IL13R α1, emerged as a clear contender for further *in vitro* studies (alongside miR-155) following the results observed in human biopsies, showing a clear significant up-regulation of this microRNA in inflamed UC colonic biopsies and inflamed UC epithelial cell isolates (***Figures 16 and 18***, respectively).

There are several bioinformatics tools available on line to predict microRNA-target interactions (see ***section 2.3***). IL13Rα1 was identified as a direct target of miR-31 using the microRNA search tool TargetScan and Microrna.org (see ***Figures 6 and 7***).

As discussed before, IL13Rα1 has been identified as a direct target of miR-155 by our group showing that reducing levels of IL13Rα1 protein leads to diminished activation of STAT6 and regulating the M2 phenotype in human macrophages (Martinez-Nunez, Louafi et al. 2011).

Figure 19 shows a reminder of the predicted binding site for miR-31 and miR-155 in the 3'UTR of IL13Rα1.

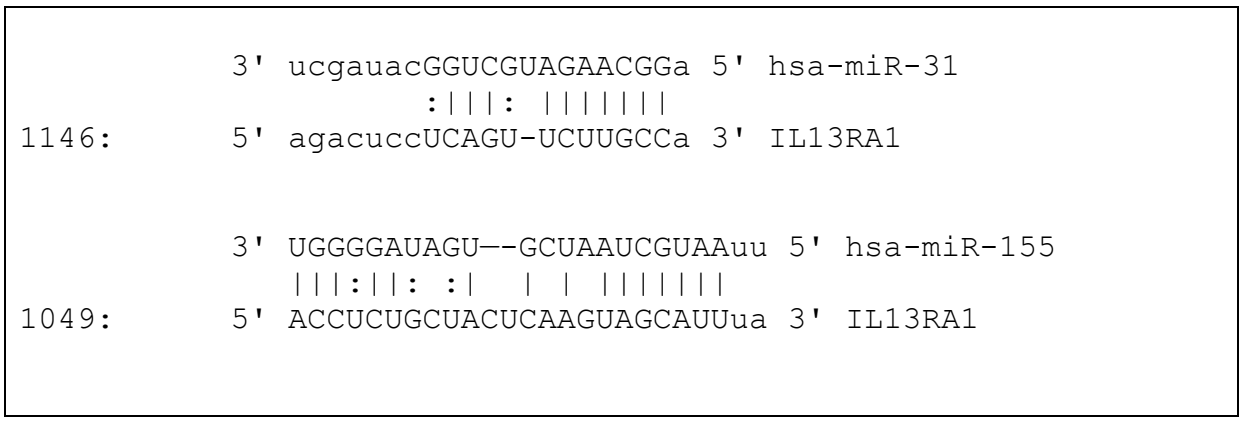


Figure 19. Schematic interaction of miR-31 and miR-155 with IL13Rα1 3'UTR.

Our patient data shows that miR-31 and mir-155 were significantly up-regulated in active UC as compared to unaffected inactive UC and normal controls (***Figure 16***). IL13Rα1 was found to be down-regulated in inflamed UC biopsy samples and inflamed epithelial cell isolates from active UC (***Figures 15 and 17***, respectively).

Fasseu *et al.* demonstrated, as discussed before, differential expression of several microRNAs in IBD in inflamed and inactive samples and miR-31 was elevated in inflamed UC and also dysregulated in CD (Fasseu, Treton et al. 2010).

As previously mentioned, Interferon-β-1a, located in the same region of chromosome 9 as miR-31, has been shown to exhibit therapeutic effects inducing remission in a subset of patients with UC via SOCS proteins (Mannon, Hornung et al. 2011). MiR-31 has been found to have a wide variety of other predicted targets highlighting the potential importance of this microRNA in the regulation of the immune response by targeting FOXP3, or HDAC1, HDAC2 and HDAC9 (Rouas, Fayyad-Kazan et al. 2009). Being able to down-regulate the activation of STAT6 by the HDAC inhibitor SAHA in UC (Rosen, Frey et al. 2011) is an interesting finding considering that miR-31 targets HDAC1, HDAC2 and HDAC9 (Rouas, Fayyad-Kazan et al. 2009).
E-selectin, which recruits neutrophils to be trans-located to a site of inflammation, is targeted by TNF-induced miR-31 and was shown to inhibit neutrophil adhesion to endothelial cells suggesting that miR-31 has a potential role in anti-inflammatory therapy (Suarez, Wang et al. 2010).
Moreover miR-31 expression has been shown to increase in a stepwise fashion as tissue goes from normal to chronically inflamed to actively inflamed to neoplastic (Olaru, Selaru et al. 2011).

All these findings of the involvement of miR-31 in vital processes of inflammation and immunology highlight the potential importance of miR-31 in IBD.

In our hypothesis we asked the question as to whether miR-31 and miR-155 are potentially responsible for this decrease of IL13Rα1 in an attempt to reduce inflammation caused by IL-13 and the activation of the STAT6 pathway.

MiR-31, together with miR-155, was thought to be a strong candidate for further experiments due to its proven dysregulation in UC (Fasseu, Treton et al. 2010, Takagi, Naito et al. 2010) and its major role in the inflammation and colorectal cancer axis in UC (Olaru, Selaru et al. 2011). Interestingly, it's predicted binding site on the IL13Rα1 3'UTR is proximal to that of miR-155 (***Figure 19***), making it an interesting prospect to investigate a possible collaboration between miR-31 and miR-155.

5.1.2 Dual Luciferase system - direct targeting of IL13Rα1 by miR-31 and miR-155

The targeting of IL13RA1 3'UTR by miR-31 was analysed co-transfecting wild type (WT) or mutated miR-31 binding site (MUT) (see ***2.11.2***) reporter constructs with our miR-31 over expressing vector (see ***Figure 8***) in HeLa cells and employing Dual Luciferase Assay (see 3.14 for Dual Luciferase Assay and 3.14.2 for the transfection protocol). ***Figure 9*** shows the predicted pairings between each site in the 3'UTR of IL13RA1 in the wild type (WT) and mutant (MUT) versions.

Figure 20 shows the results of 3 independent experiments. Co transfection of miR-31 with the wild type construct led to a significant reduction of the renilla luciferase activity by 50% (***Figure 20***, WT black bar). Mutation of the predicted binding site for miR-31 abolished miR-31 effects (***Figure 20***, MUT black bar). Statistical analysis was performed using t-test with GraphPad Prism version 5.00 for Windows.

This experiment demonstrates that miR-31 directly binds to the 3'UTR of IL13RA1 and maps its binding site to position 1146. It also provides a direct molecular link in our findings in our patient data showing a reduction in IL13Rα1 in active disease with high levels of miR-31.

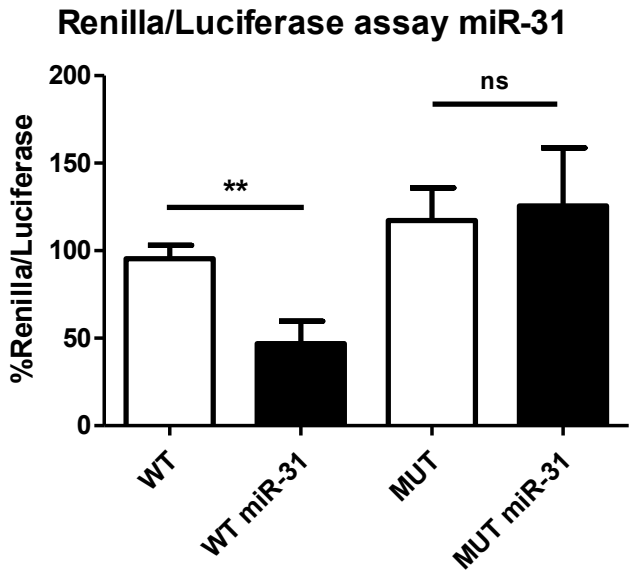


Figure 20. Mir-31 directly targets the 3'UTR of IL13Rα1. Renilla luciferase reporter constructs (WT is intact IL13Rα1 3'UTR and MUT is mutated for the predicted binding site for miR-31) were co transfected with empty pCDNA3.1 (white bars, -) or pCDNA3.1-miR-31 (black bars). Renilla reporter for IL13Rα1 3'UTR showed a significant drop when miR-31 was co-transfected (black bars, WT). MiR-31 over expression had no effect on the mutant version (black bars, MUT) showed no effect. Firefly luciferase was used as normalizer. **:P-value<0.01(P-value=0.0086).

5.2 Functional role of miR-31 and miR-155 in an *in vitro* colonic epithelial cell model (HT-29 cells)

5.2.1 Introduction

As pointed out in the introduction, disruption of the mucosal barrier by the direct toxic effects of IL-13 and the presence of its main producer NKT-cells are the hallmarks of UC. It leads to increased mucosal permeability as first shown in murine studies (Heller, Fuss et al. 2002). This was confirmed after by an *in vitro* study showing that IL-13 decreases the mucosal resistance in HT-29 cells and also increases the production of claudin-2, which leads to increased epithelial barrier permeability in UC (Heller, Florian et al. 2005).

HT-29 cells are a cell line derived from a human colon adenocarcinoma and exhibit characteristics of normal intestinal epithelium such as epithelial polarity, presence of the actin binding protein villin and the occurrence of an enterocytic differentiation (Chantret, Barbat et al. 1988). HT-29 cell line has been used for *in vitro* experiments in IBD research in relation to IL-13 (Heller, Fromm et al. 2008, Mandal and Levine 2010). It has also been shown to expresses IL-4Rα, IL13Rα1, and IL-13Rα2 by RT-PCR (Blanchard, Durual et al. 2004), and therefore due the availability of this cell line (Dr Chris Piccard) was chosen for *in vitro* epithelial experiments. *Ex vivo* models of human colonic epithelium have had limited success, due to their short viability (Dame, Jiang et al. 2013).

First the expression of the genes of interest in this epithelial cell line was determined to establish the expression of the IL-13 and TGF-β dependent genes, some of which were found deregulated in our UC study population (***Figure 13***). Of particular interest was the expression profile of SOCS1 and IL13Rα1 as these have been demonstrated targets of miR-31 (IL13Rα1) and miR-155 (IL13Rα1, SOCS1).

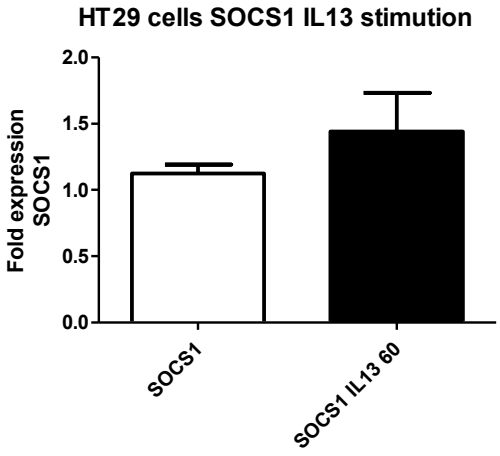
We employed ΔΔCt method to calculate the fold induction of mRNA and microRNA expression (***see 2.18.1*** for ΔΔCt method principle). As a brief note, higher C_T values (later values) mean late amplification. In un-stimulated HT-29 cells SOCS1 mRNA showed a C_T value of about 30 and IL13Rα1 mRNA of about 25. SERPINE1 was detected around 26 of C_T and CCL26 at about 34 in un-stimulated cells. Other genes measured in patient samples such as CCL18, MMP9 and TNF-α

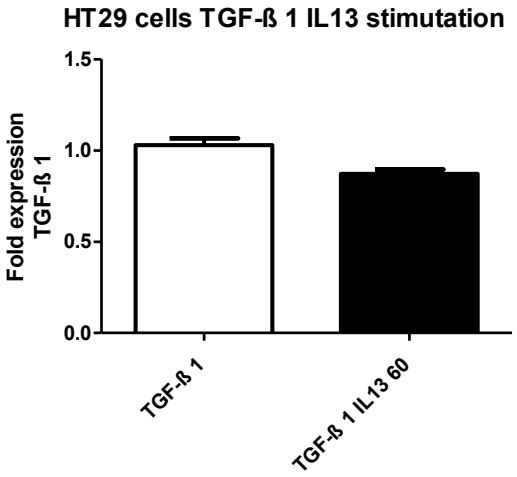
were found not to be expressed by HT-29 cells (data not shown). Expression of miR-31 and miR-155 in HT-29 cells was found at C_T values of about 22 and 25 respectively, suggesting that these microRNAs are highly expressed in these cells (data not shown).

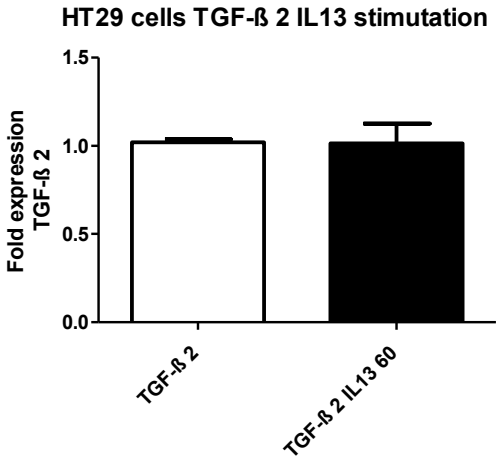
5.2.2 Expression profile of potential target genes in HT-29 cells and their response to IL-13 and TGF-β

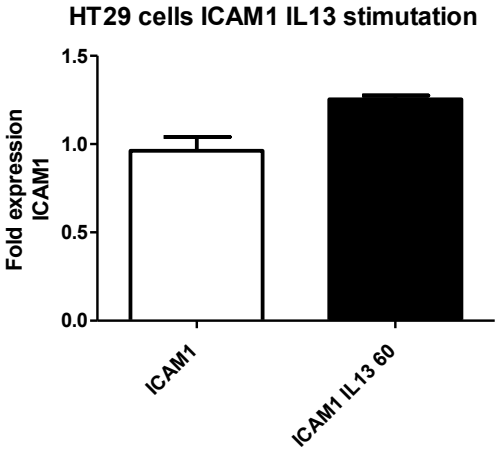
To determine potential gene targets for *in vitro* experiments in HT-29 cells, a host of IL-13 and TGF-β dependent genes were tested to study their expression and response to IL-13 and TGF-β. Some of the genes chosen were shown to be differentially expressed in the colonic tissue samples in normal controls, unaffected inactive and active UC such as CCL18, SOCS1, SERPINE1, MMP9, CCL26 and IL13Rα1 (***Figure 13***). SOCS1 and IL13Rα1 were also found to be differentially expressed in colonic epithelial cells isolated from normal controls, unaffected inactive UC and active UC (***Figure 17***).

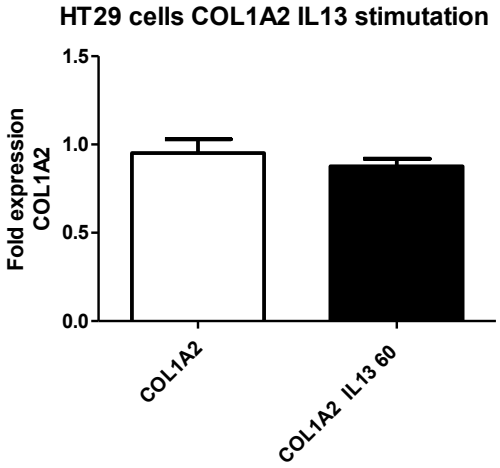
Testing their expression in this cell line was the basis for further *in vitro* functional studies. Furthermore, we looked at the expression and response of variety of other genes regulated by the IL-13 and TGF-β stimulation (***Figures 21 and 22***, respectively) to expand on potential targets for functional studies and determine their response to IL-13 and TGF-β stimulation. Expression of TGFβ 1 and 2, ICAM1, COL1A2 and SMAD2 in the TGF-β pathway was tested (***Figures 21 and 22***). In the IL-13 pathway we assayed the expression of IL-8, CCL5, CCL18 and CCL22 in addition to the genes tested on our colonic biopsies (***Figures 21 and 22***). These genes were also chosen based on previous work in our laboratory on respiratory epithelium and human macrophages. For the IL-13 stimulation 60ng/ml of IL-13 was used and for stimulation with TGF-β 5nM was used. Cells were collected at 24 hours. CCL18 and MMP9 were not expressed by HT-29 cells.

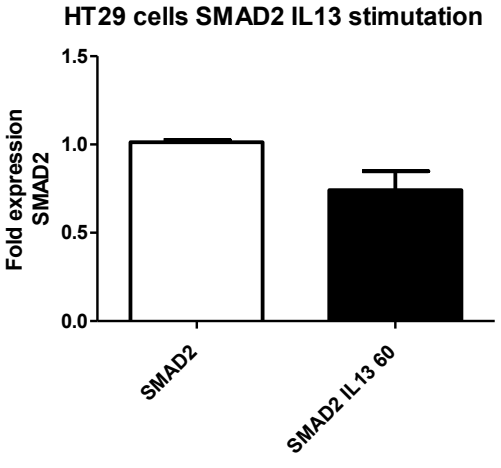

HT29 cells SOCS1 IL13 stimution
Fold expression SOCS1
2.0
1.5
1.0
0.5
0.0
SOCS1
SOCS1 IL13 60


HT29 cells TGF-ß 1 IL13 stimutation
Fold expression TGF-ß 1
1.5
1.0
0.5
0.0
TGF-ß 1
TGF-ß 1 IL13 60


HT29 cells TGF-ß 2 IL13 stimutation
Fold expression TGF-ß 2
1.5
1.0
0.5
0.0
TGF-ß 2
TGF-ß 2 IL13 60


HT29 cells ICAM1 IL13 stimutation
Fold expression ICAM1
1.5
1.0
0.5
0.0
ICAM1
ICAM1 IL13 60


HT29 cells COL1A2 IL13 stimutation
Fold expression COL1A2
1.5
1.0
0.5
0.0
COL1A2
COL1A2 IL13 60


HT29 cells SMAD2 IL13 stimutation
Fold expression SMAD2
1.5
1.0
0.5
0.0
SMAD2
SMAD2 IL13 60

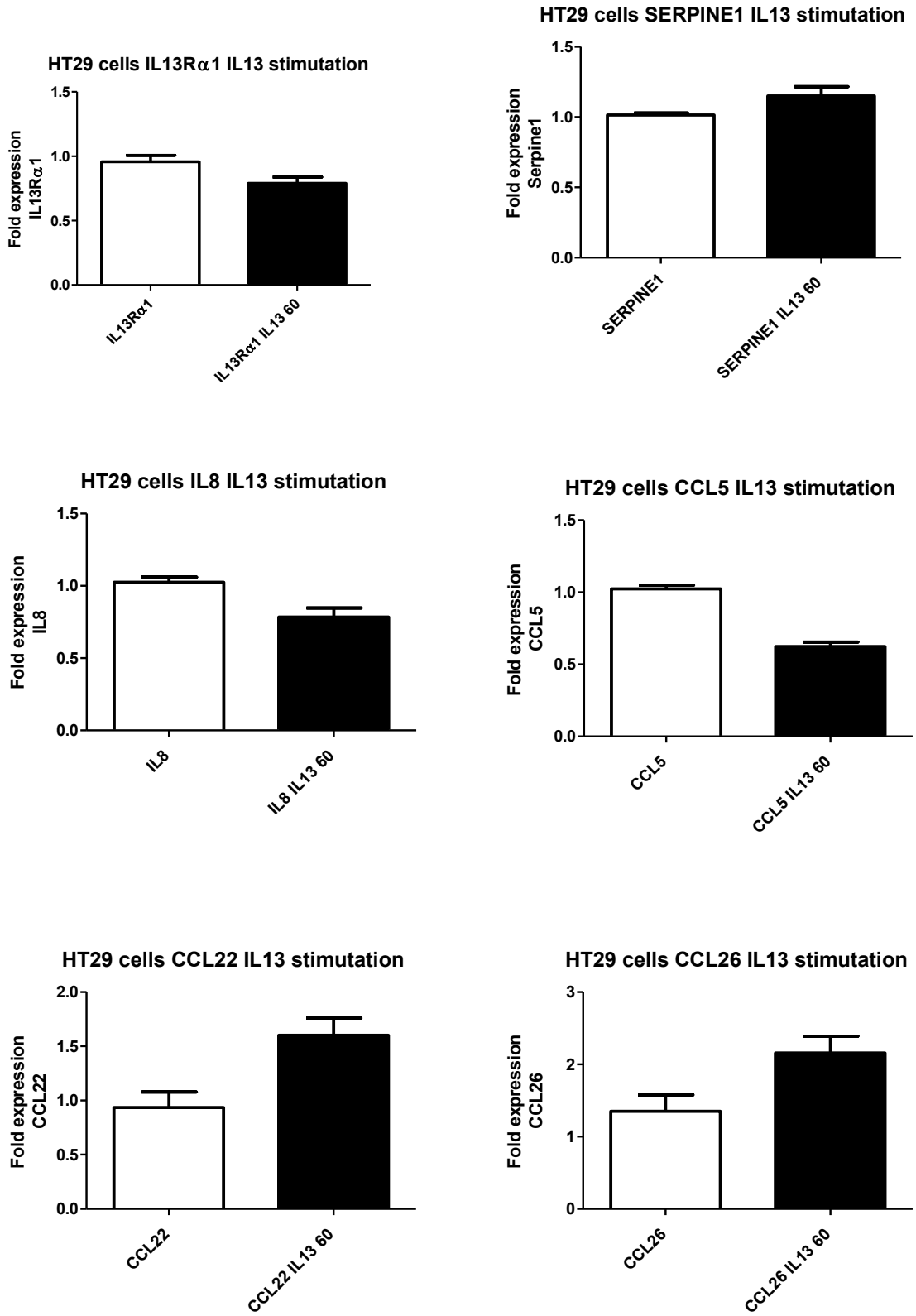
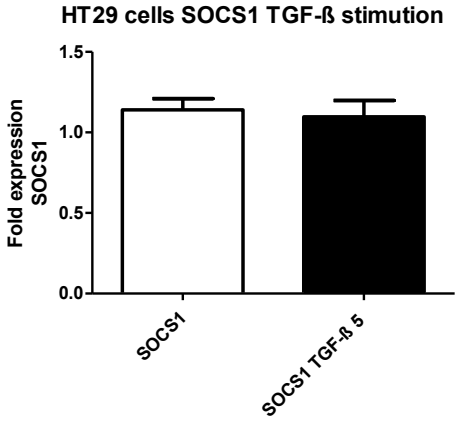
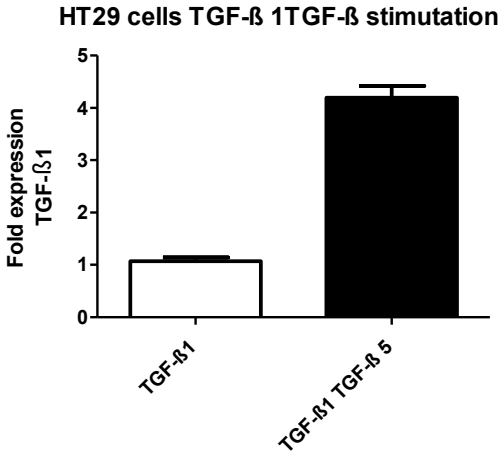
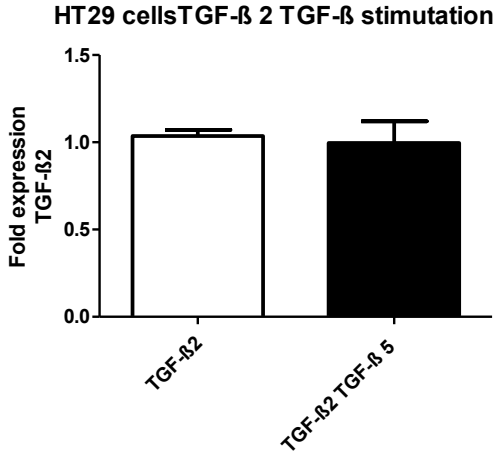
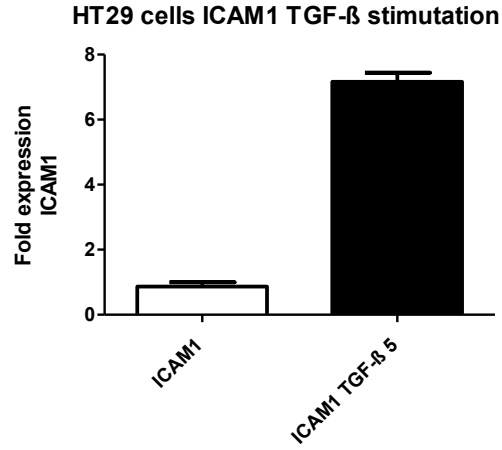


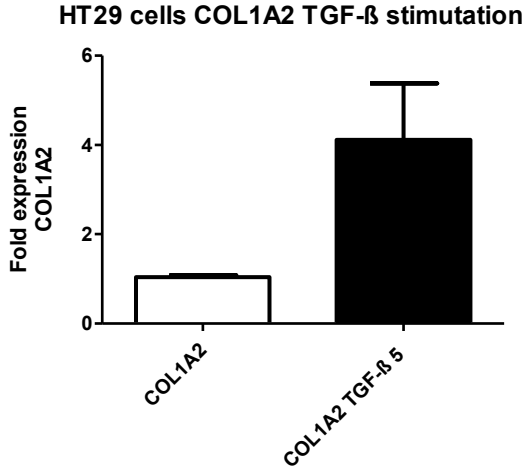
Figure 21. Expression of the chosen genes and their reaction to IL-13 stimulation in HT-29 cells. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

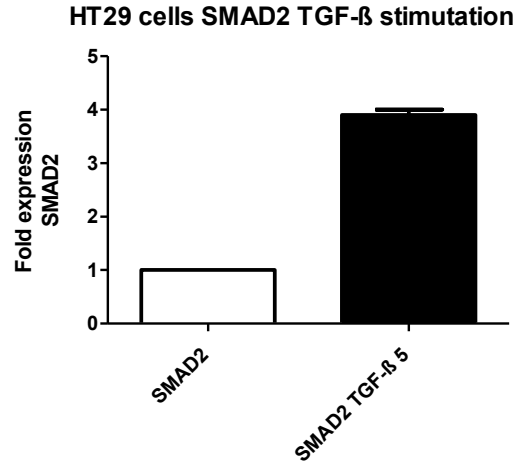

HT29 cells SOCS1 TGF-ß stimution
Fold expression SOCS1
1.5
1.0
0.5
0.0
SOCS1
SOCS1 TGF-ß 5


HT29 cells TGF-ß 1TGF-ß stimutation
Fold expression TGF-ß1
5
4
3
2
1
0
TGF-ß1
TGF-ß1 TGF-ß 5


HT29 cellsTGF-ß 2 TGF-ß stimutation
Fold expression TGF-ß2
1.5
1.0
0.5
0.0
TGF-ß2
TGF-ß2 TGF-ß 5


HT29 cells ICAM1 TGF-ß stimutation
Fold expression ICAM1
8
6
4
2
0
ICAM1
ICAM1 TGF-ß 5


HT29 cells COL1A2 TGF-ß stimutation
Fold expression COL1A2
6
4
2
0
COL1A2
COL1A2 TGF-ß 5


HT29 cells SMAD2 TGF-ß stimutation
Fold expression SMAD2
5
4
3
2
1
0
SMAD2
SMAD2 TGF-ß 5

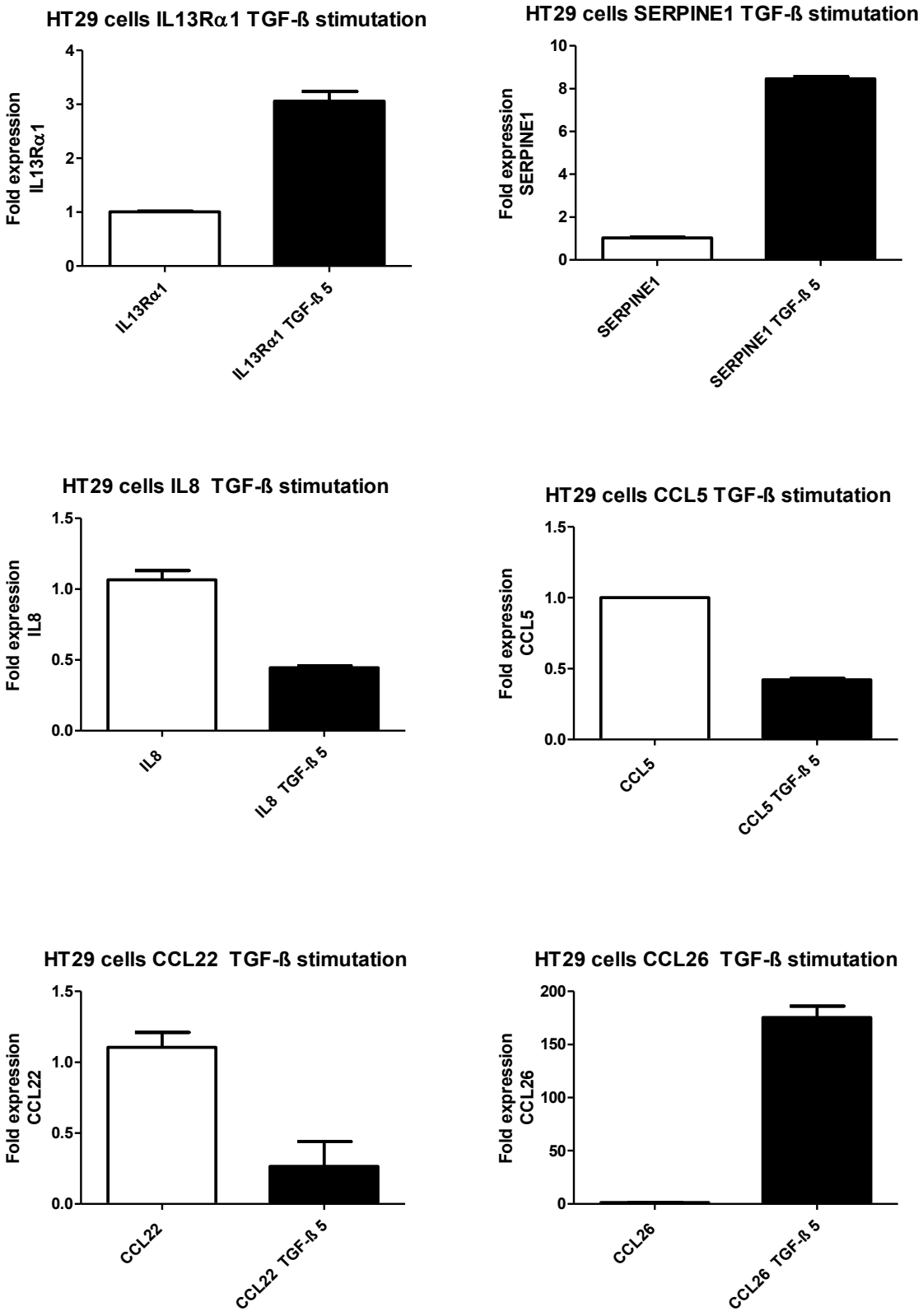


Figure 22. Expression of the chosen genes and their reaction to TGF-ß stimulation in HT-29 cells. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

The observed response to IL-13 stimulation was mild (***Figure 21***). SOCS1 and CCL26 mRNA showed an increase of mRNA after IL-13 stimulation, and these two genes were chosen alongside IL13Rα1, the main focus of our studies showing in the luciferase experiment that it is a direct target for miR-31 (***Figure 20***) and targeted by miR-155 (Martinez-Nunez, Louafi et al. 2011), for further investigations in HT-29 cells.

Response of TGF-β dependent genes to TGF-β was generally more pronounced (***Figure 22***) compared to the response of IL-13 pathway genes after IL-13 stimulation.

In view of the fact that the microRNAs of interest miR-31 (see ***Figure 20****)* and miR-155 have been shown to target IL13Rα1 (Martinez-Nunez, Louafi et al. 2011) we decided to concentrate on the IL-13 pathway genes SOCS1 (Mannon, Hornung et al. 2011, Rosen, Frey et al. 2011) and CCL26 (Blanchard, Durual et al. 2005, Manousou, Kolios et al. 2010) both of which have been shown to be of importance in UC, alongside our main focus of interest IL13Rα1.

5.2.3 Transfection efficiency of HT- 29 cells with anti-miR-31 and 155 at 24 and 48 hours

To determine the transfection efficiency of HT-29 cells, we transfected cells with anti-miR-31 (100nM) and anti-miR-155 (100nM) to assess the knock down effect on the expression of these microRNAs using different concentrations of the transfection agent Interferin (see ***section 2.11.1***). MicroRNA levels were measured by RT-qPCR (see ***sections 2.16, 2.17 and 2.18***).

Figure 23 shows the reduction of expression of miR-31 and miR-155 in HT-29 cells in the presence of anti-miR-control and anti-miR-31 and anti-miR-155 with different concentrations of Interferin 24h post transfection. An almost complete knockdown of miR-31 and miR-155 was achieved with 100nM of the respective microRNA with the minimum concentration of the transfection agent Interferin, 5μL per 600μl total volume of media.

This experiment confirmed that HT-29 cells are susceptible to transfection with anti-microRNAs, providing a good *in vitro* epithelial cell model for further functional studies investigating the modulation of IL-13 pathway by miR-31 and miR-155 through IL13Rα1.

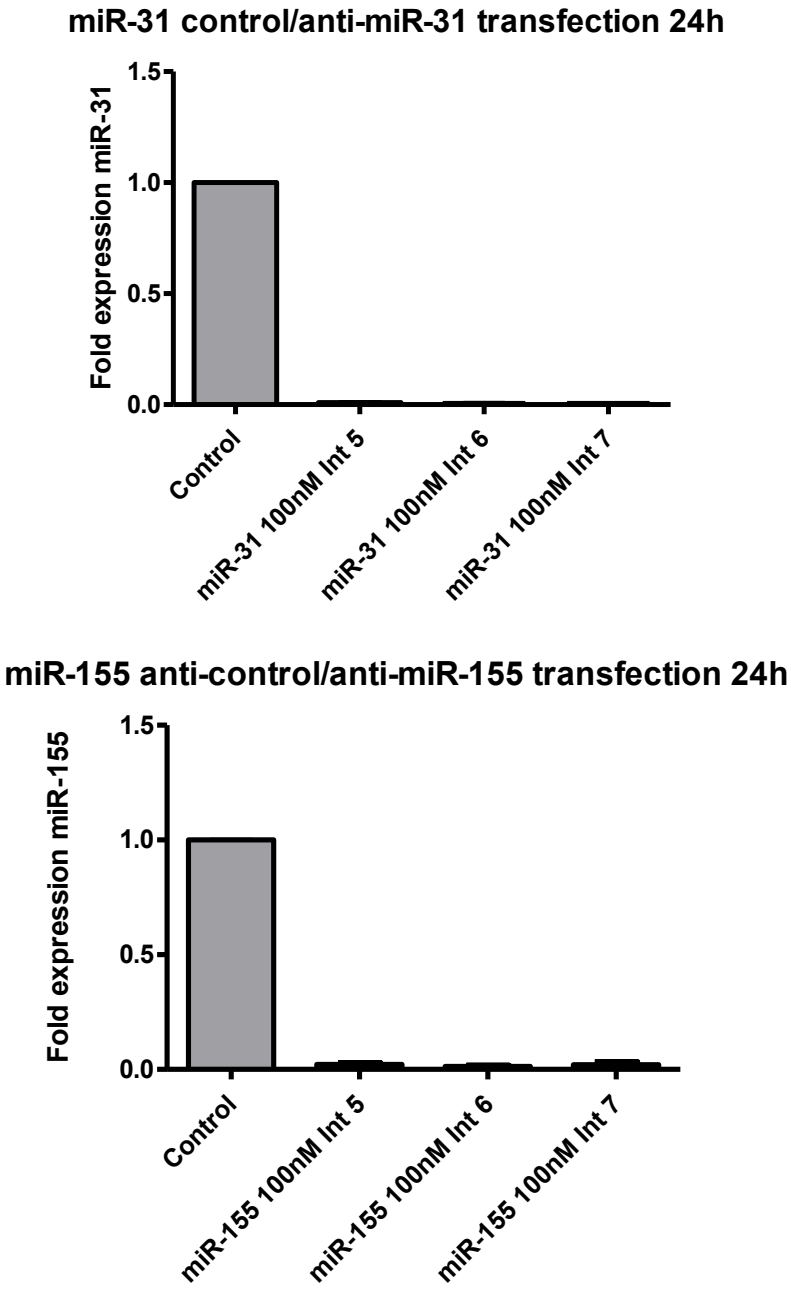


Figure 23. Reduction of expression of miR-31 and miR-155 in HT-29 cells at different concentrations of Interferin 24h post transfection. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

5.2.4 IL-13 dose response experiment in HT 29 cells

To establish the responsiveness of HT-29 cells to IL-13, cells were stimulated with various doses of IL-13 and SOCS1 expression was measured at 12, 24 and 48 hours. The mRNA expression of SOCS1 was assessed by RT-qPCR (see ***sections 2.16, 2.17 and 2.18***). No major over-expression of SOCS1 was detected using 25 or 50ng/ml of IL-13 at 12, 24 or 48 hours. A response doubling the amount of SOCS1 mRNA was seen after 48 hours post stimulation with 100ng/ml of IL-13 (***Figure 24***)

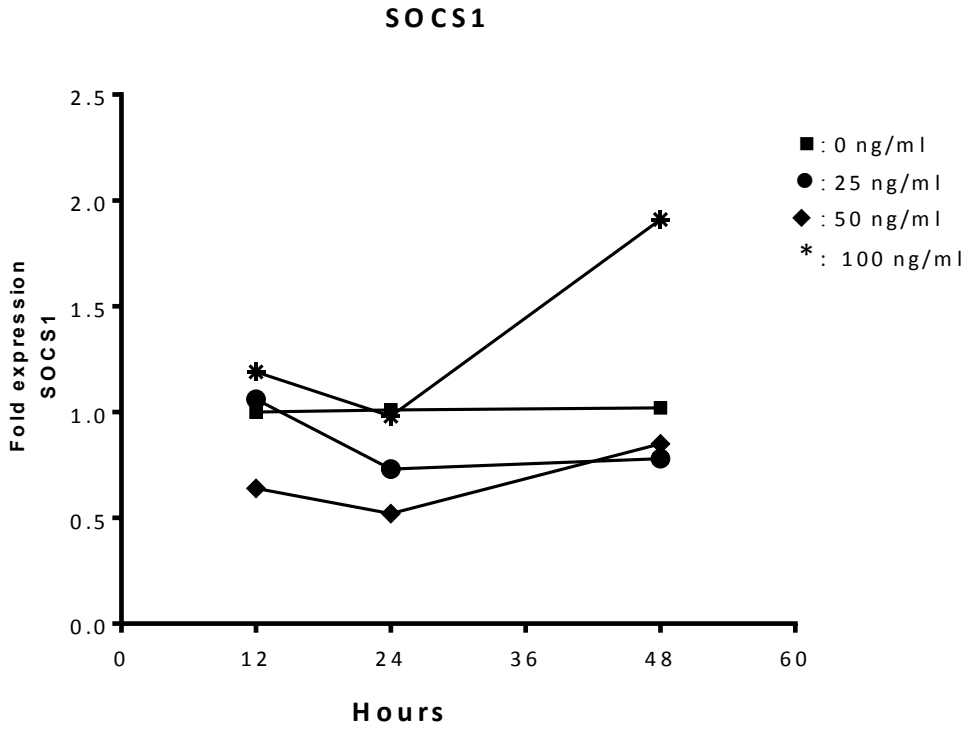


Figure 24. Dose dependent response to IL-13of SOCS1 at 12, 24 and 48 hours. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

The stimulation observed after IL13 treatment showed only a slight (2 fold) rise of SOCS1 mRNA at 48 hours. Due to the poor response a detailed literature research was undertaken. Published work from Hebenstreit *et al* in a human lung epithelial cell line showed that SOCS1 mRNA levels peaked after 1h with an induction up to 5- to 7-fold increased levels in comparison with un-

stimulated cells, when stimulated with IL-13 (Hebenstreit, Luft et al. 2003). Therefore, our experiments using IL-13 stimulation were repeated using different time points.
IL-13 stimulation was undertaken at 1 and 8 hours using the same brand of IL-13 at 100ng/ml. ***Figure 25*** shows the results of this experiment. A small response was seen at 1 hour but it was not as high as shown by Hebenstreit et al (Hebenstreit, Luft et al. 2003). No response of SOCS1 was observed at 8 hours.

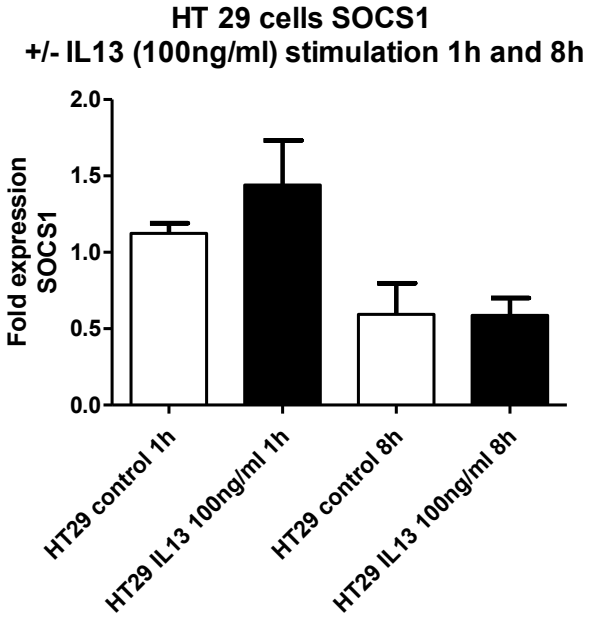


Figure 25. SOCS1 mRNA expression response after stimulation with IL-13 100ng/ml at 1 and 8 hours. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls at 1 hour. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

In view of the difficulties encountered in this specific IL-13 stimulation model and IL-13 stimulation of epithelial cells and THP-1 cells had proved difficult before, it was agreed to concentrate on transfection of cell lines with microRNAs and to purchase a new brand of IL-13 once the transfection models were working.

5.2.5 MiR-31 and miR-155 expression in HT-29 cells post stimulation with IL-13

To establish whether the stimulation with IL-13 significantly influenced the expression of miR-31 and miR-155, expression of these microRNAs was assessed during different time points after IL-13 stimulation (100ng/ml). MiR-31 levels were practically unaltered (***Figure 26***) and miR-155 doubled at 48 hours (***Figure 27***). This experiment was designed to determine whether microRNA levels can be induced after IL-13 stimulation.

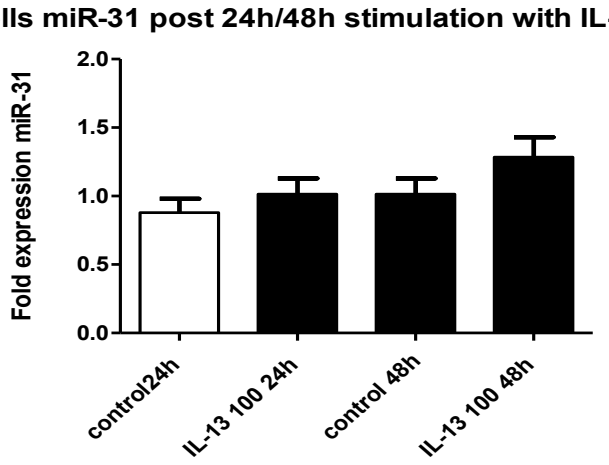


Figure 26. MiR-31 expression levels upon IL-13 stimulation of HT-29 cells. MiR-31 does not significantly change during the course of the IL-13 stimulation over 48 hours as determined by RT-qPCR. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls at 24h. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

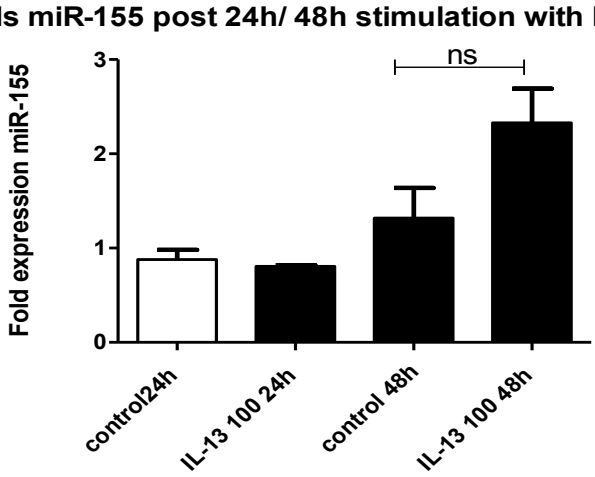


Figure 27. MiR-155 expression levels upon IL-13 stimulation of HT-29 cells. MiR-155 expression shows a twofold increase at 48 hours post stimulation with IL-13 as determined by RT-qPCR. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls at 24h. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

5.2.5.1 Discussion

The results show that miR-31 and miR-155 levels are not significantly altered after IL-13 stimulation (***Figures 26*** and ***27***, respectively). The rise miR-155 at 48 hours will not influence our results as we are not planning to stimulate cells for more than 24 hours. The peak of SOCS1, an IL-13 induced gene, is at 1 hour and gets progressively less pronounced towards 24 hours as described above (Hebenstreit, Luft et al. 2003) and future experiments will have the time points adjusted to this finding.

5.2.6 Transfection of HT-29 cells with pre-miR-31 and pre-miR-155 and a combination of pre-miR-31 and pre-miR-155 to assess effect on IL-13 dependent genes

It has already been established that HT-29 cells can be successfully transfected with microRNAs, evidenced by a complete knockdown of miR-31 and miR-155 after transfection with anti-miR-31 and anti-miR-155 (***Figure 23***). We were then interested in assessing the effect of pre-miRs on the expression of SOCS1 and IL13Rα1, in an IL-13-dependent manner. Three further genes downstream in the IL-13 pathway were also assayed. SERPINE1, which was shown to be raised in our tissue samples (***Figure 13***), CCL5 and CCL22, differentially expressed in HT-29 cells after IL-13 stimulation (CCL5 was decreased and CCL22 was increased after IL-13 stimulation) (***Figure 21***). mRNA levels were determined RT-qPCR as described in ***sections 2.16, 2.17 and 2.18***.

Transfection with pre-miR-31 at 100nM, 75nM, 50nM and 25nM with 5μl of Interferin (for protocol see ***section 2.11.1***) was undertaken. Samples were stimulated with IL-13 100ng/ml or un-stimulated.

Results revealed a microRNA-dose dependent down-regulation of SOCS1 that reached its maximum (about 40%) at a dose of 100nM of pre-miR-31 (24 hour transfection) and up to about 60% at 75nM compared to the un-stimulated sample 48 hours post transfection (See ***Figure 28***).

A very similar dose dependent decrease of IL13Rα1 mRNA was observed after pre-miR-31 transfection, with a reduction of about 60% at a dose of 100nM and 40% at a dose of 75nM compared to the control sample (***Figure 29***).

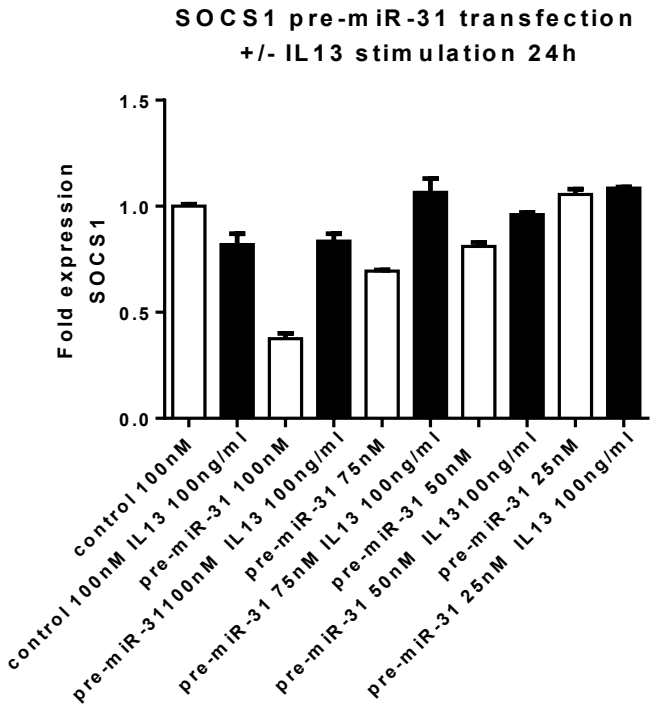


Figure 28. Dose dependent down-regulation of SOCS1 by miR-31. SOCS1 mRNA down regulation reached its maximum (about 40%) at a dose of 100nM of pre-miR-31 24h post transfection. SOCS1 down regulation of about 60% was observed at a dose of 75nM pre-miR-31. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

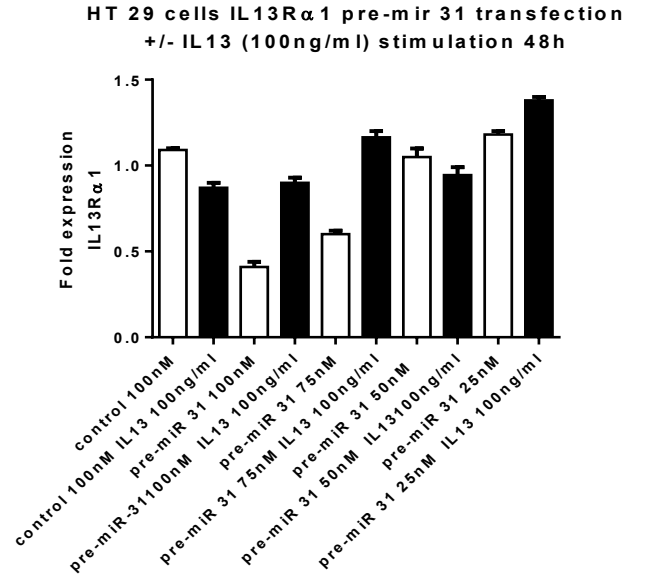


Figure 29. Dose dependent decrease of IL13Rα1 mRNA after pre-miR-31 transfection. IL13Rα1 mRNA expression shoed a reduction of about 60% at a dose of 100nM and 40% at a dose of 75nM as compared to the control sample. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

No dose dependent effect on the expression of CCL5, CCL22 and SERPINE11 was observed after transfection with pre-miR-31 (***Figure 30***) irrespective of IL-13 stimulation and therefore these genes were not tested in the following experiment using pre-miR-155, in order to focus on common targets of both microRNAs.

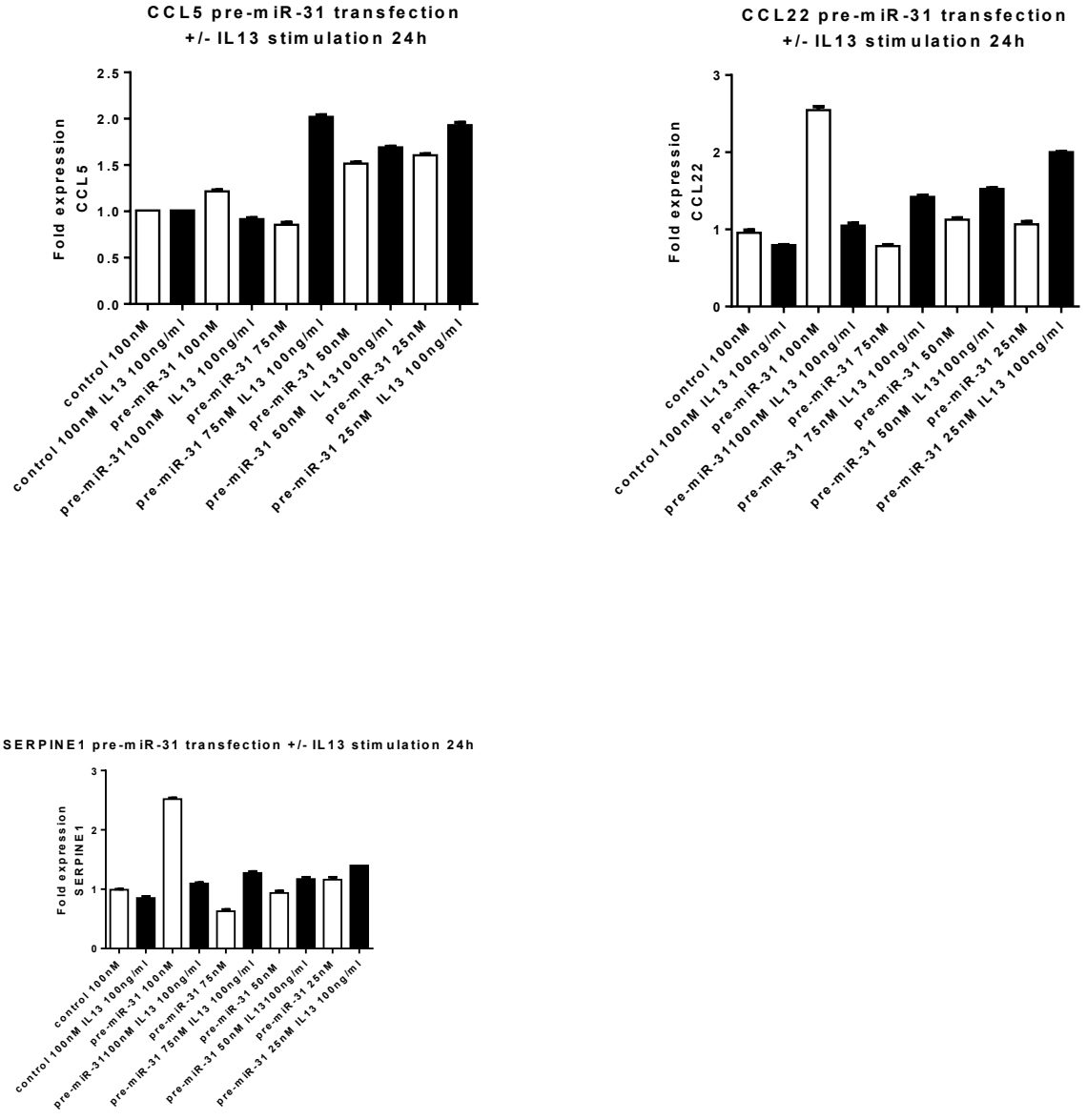


Figure 30. mRNA levels of CCL5, CCL22 and SERPINE1 upon miR-31 transfection. No dose dependant effect on the expression of CCL5, CCL22 and SERPINE1 was observed after the transfection with pre-miR-31. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls at 24h. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

Figure 31 and ***Figure 32*** demonstrate the effect of pre-miR-155 in SOCS1 and IL13Rα1 mRNA expression levels in an IL-13 dependent manner. Experiments using pre-miR-155 in HT-29 cells showed a less dramatic effect at a dose of 100nM on the expression of mRNA of SOCS1 and IL13Rα1 mRNA. SOCS1 was reduced to about 70% as compared to the control sample. IL13Rα1 mRNA levels were reduced by about 20% as compared to the control sample (***Figure 31*** and ***Figure 32,*** respectively).

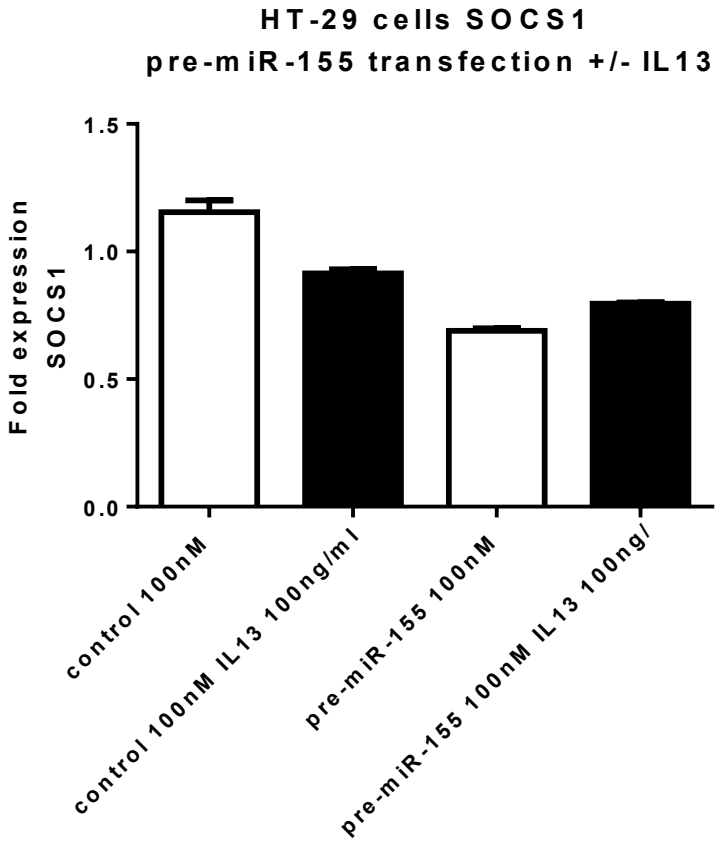


Figure 31. Effect of pre-miR-155 on SOCS1 mRNA expression. MiR-155 transfection showed reduction of SOCS1 to about 70% compared to control cells. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

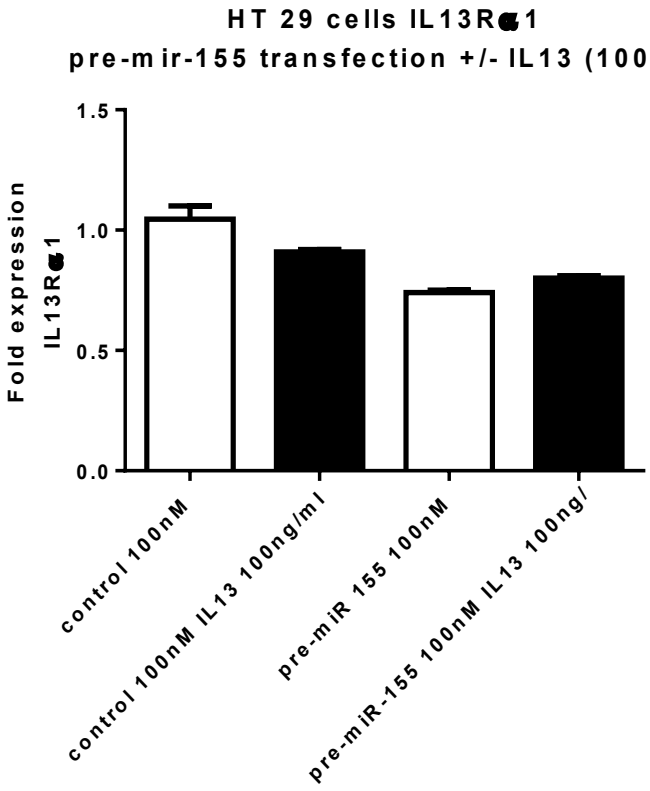


Figure 32. Effect of pre-miR-155 on IL-13Rα1 mRNA expression. MiR-155 transfection showed reduction of IL13α1mRNA by about 20% compared to control cells. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

Combination of pre-miR-31 and pre-miR-155 at a reduced dose of 25nM each was studied to assess whether these two microRNAs had a synergistic effect in reducing IL13Rα1 and subsequently also affected the expression of SOCS1 mRNA. This revealed a very interesting finding demonstrating that half of the total dose achieved more significant effects in the reduction of SOCS1 and IL13Rα1 as compared to the same dose of pre-miR-31. SOCS1 mRNA expression was reduced to about 50% and the expression of IL13Rα1 was reduced by 30% (***Figure 33*** and ***Figure 34***, respectively)**.** This indicates not only an additive effect but may well constitute a synergistic mode of action of these two microRNAs which has not been demonstrated before, but stimulation with IL-13 did again not have the desired effect.

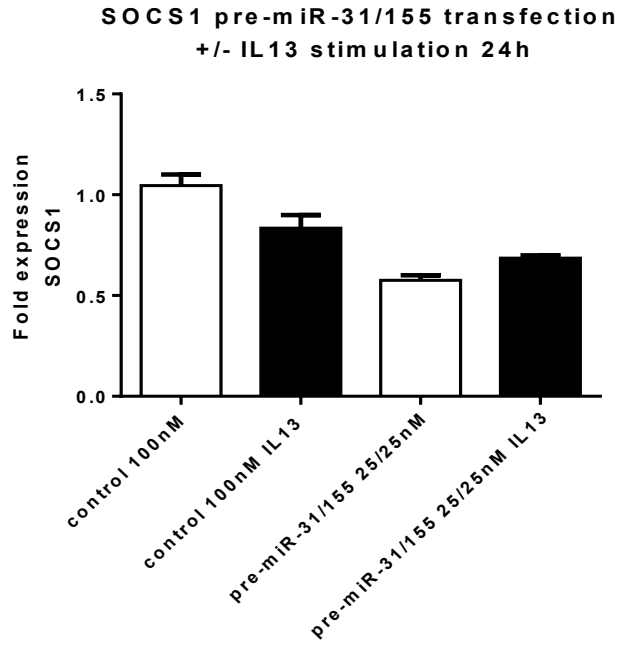


*Figure 33. Combination of pre-miR-31 and pre-miR-155 in SOCS1 mRNA levels. Mir-155 and miR-31 were transfected in HT-29 cells at a concentration of 25nM each (a quarter of the original dose). SOCS1 to about 50% compared to its control indicating a synergistic effect at this reduced dose. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*

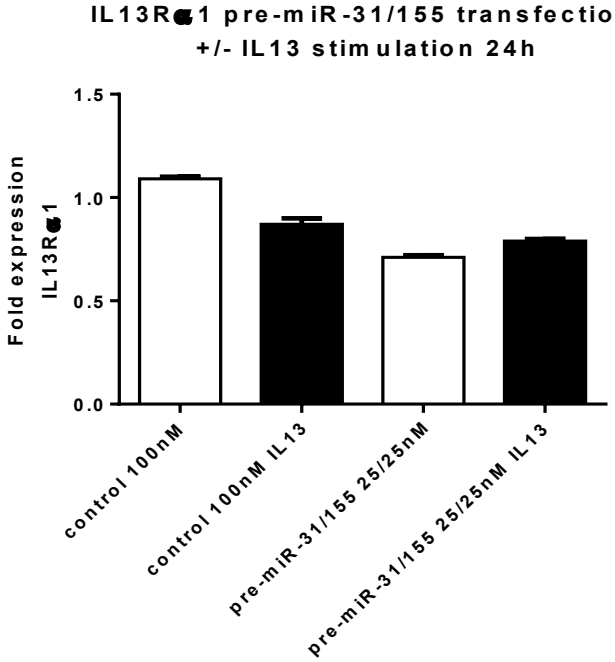


*Figure 34. Combination of pre-miR-31 and pre-miR-155 in IL-13Rα 1mRNA levels. Mir-155 and miR-31 were transfected in HT-29 cells at a concentration of 25nM each (a quarter of the original dose). IL13Rα1 mRNA levels were reduced by about 30% compared to control indicating a synergistic effect at this reduced dose. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*

5.2.7 Repeat of IL-13 stimulation with new IL-13 cytokine batch

After having observed that miR-31, miR-155 and their combination do seem to be able to reduce the mRNA expression of the IL-13 dependent genes SOCS1 and IL13Rα1 (***Figures 28, 29, 31-34)***, the response to IL-13 stimulation in previous experiments was still unsatisfactory. As previously discussed, published data from Hebenstreit *et al.* (Hebenstreit, Luft et al. 2003) suggests that IL-13 stimulation in epithelial cells should be much more pronounced, and especially SOCS1 has been shown to have a peak of expression after one hour of IL-13 treatment. The response then tails off towards 24 hours.

A new batch of IL-13 was purchased (Recombinant human IL-13 (#213-IL-005, R&D Systems Europe Ltd., Oxfordshire, UK) and IL-13 stimulation experiments in HT-29 cells were set up using doses of 25, 50 and 100ng/ml. Cells were collected at 1, 6 and 24 hours.

Using this new batch of IL13, the results obtained were similar to those published by Hebenstreit *et al*. (Hebenstreit, Luft et al. 2003). A maximum response of SOCS1 at 1 hour was shown, and increased expression of SOCS1 mRNA of 6 fold at 1 hour at the maximum dose of 100ng/ml. As previously described the effect of IL-13 wears off towards 24 hours (***Figure 35***).

CCL26 has been chosen as candidate IL-13 dependent gene as previous IL-13 stimulation experiments have indicated that its mRNA expression increases after IL-13 treatment (***Figure 21***). CCL26 is expressed in HT-29 cells and was also elevated in our active UC group (***Figure 13***), in addition it also has been shown elevated in active UC (Manousou, Kolios et al. 2010). The peak expression of CCL26 was after 24 hours in contrast to the early rise of SOCS1. After 24 hours CCL26 was raised more than 50 fold at the maximum dose of 100ng/ml of IL-13 (***Figure 36***).

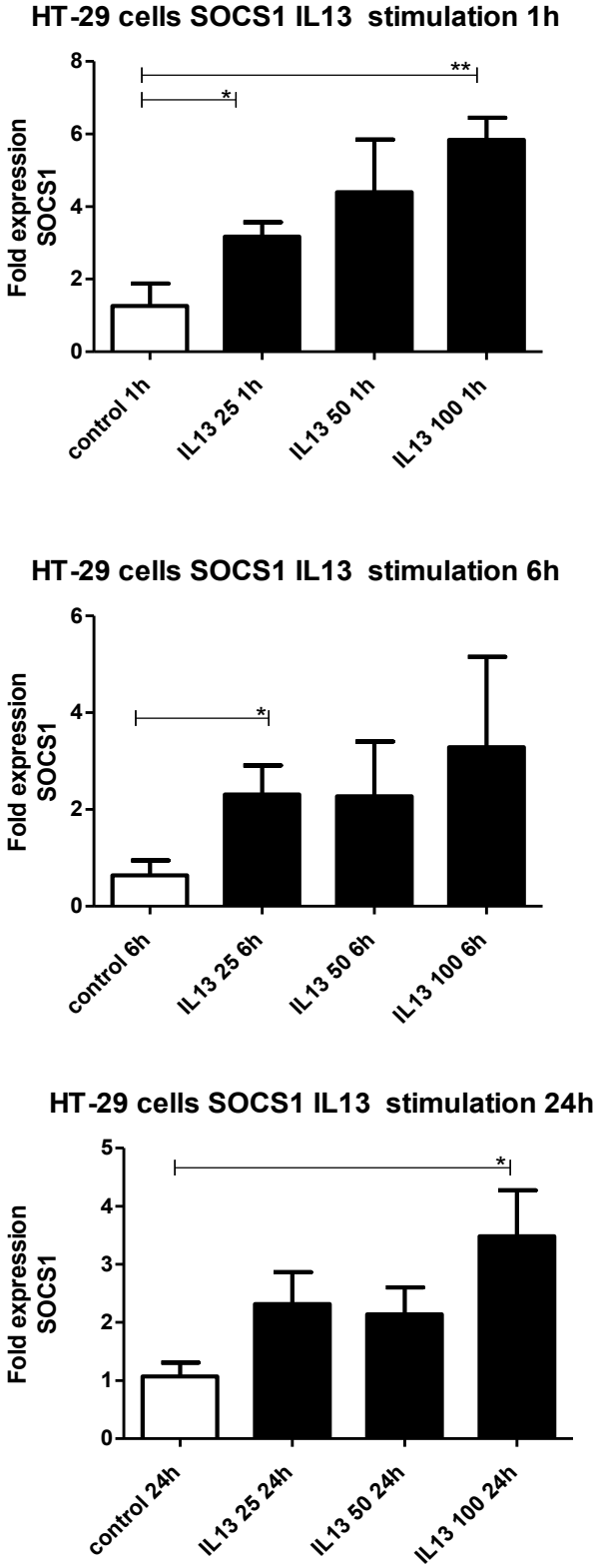


Figure 35. SOCS1 mRNA expression in HT-29 cells at 1, 6 and 24 hours after stimulation with 25, 50 and 100ng/ml of IL-13. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

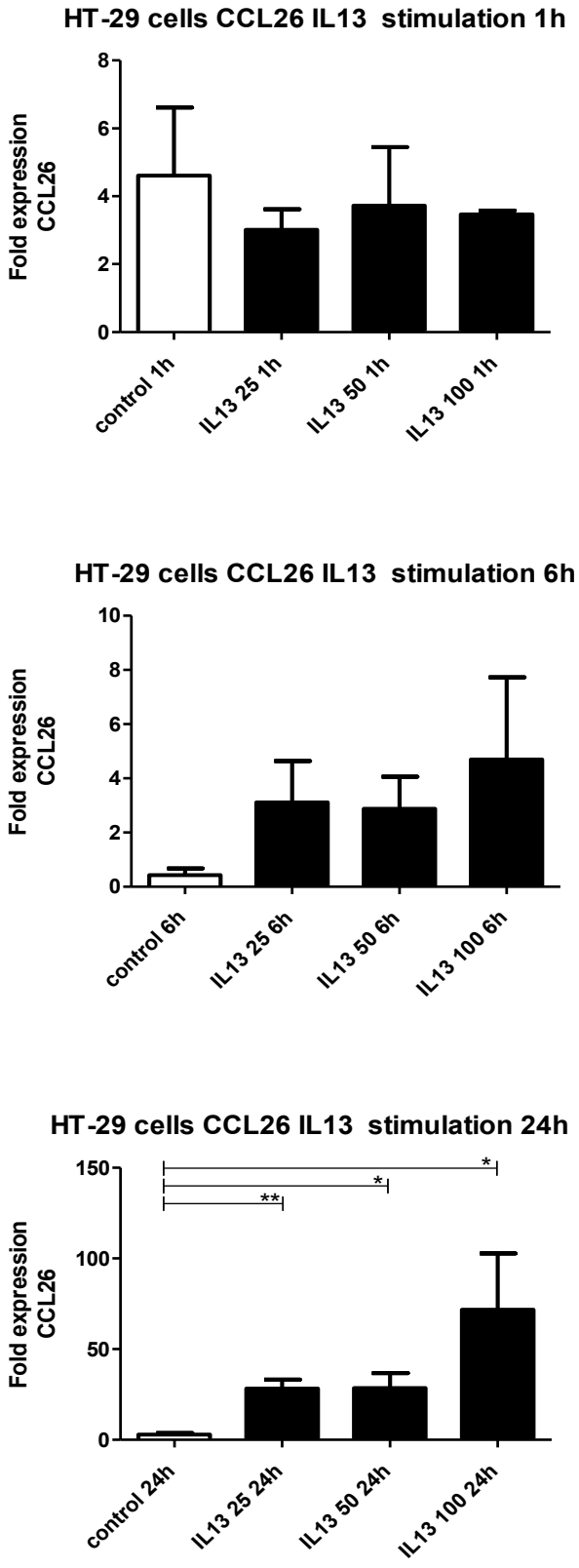


*Figure 36. CCL26 mRNA expression in HT-29 cells at 1, 6 and 24 hours after stimulation with 25, 50 and 100ng/ml of IL-13. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*

With the results of the IL-13 stimulation in keeping with the literature (Hebenstreit, Luft et al. 2003), transfections with pre-miR-31 and pre-miR-155 or their combination were repeated to investigate as to whether the IL-13 response of the candidate genes SOCS1 and CCL26 could be diminished by these microRNAs targeting IL13Rα1. IL-13 at 100ng/ml was the most potent and reliable dose for stimulation of mRNA expression of the genes of interest (***Figures 35*** and ***36***), and this dose was therefore used in future experiments.

Firstly, the transfection of HT-29 cells with pre-miR-31 using the new IL-13 agent was repeated. A titration of pre-miR-31 was applied using 25nM, 50nM and 100nM to find the optimal dose of transfection. Samples were collected at 1, 6 and 24 hours. mRNA levels of SOCS1 and CCL26 were measured (see ***2.16, 2.17*** and ***2.18***) in IL-13 stimulated and un-stimulated cells. IL13Rα1 mRNA was measured without IL-13 stimulation as it had not shown a response to this cytokine in previous experiments (***Figure 21***).

Results showed that the response of SOCS1 mRNA expression to IL-13 could be reduced at 6 hours in the two higher doses of pre-miR-31 (50nM and 100nM), but it did not reach statistical significance. At 24 hours the IL-13-dependent response of SOCS1 was significantly reduced in the 2 higher doses of the miR-31 transfection (***Figure 37***).

CCL26 mRNA levels did not show a significant response in the reduction of its mRNA expression after stimulation with IL-13 at 1 or 6 hours. It peaked at 24 hours in line with the previous IL-13 stimulation (***Figure 36***). CCL26 was significantly reduced 50ng/ml and at the highest dose of pre-miR-31 post IL-13 stimulation (***Figure 38***).

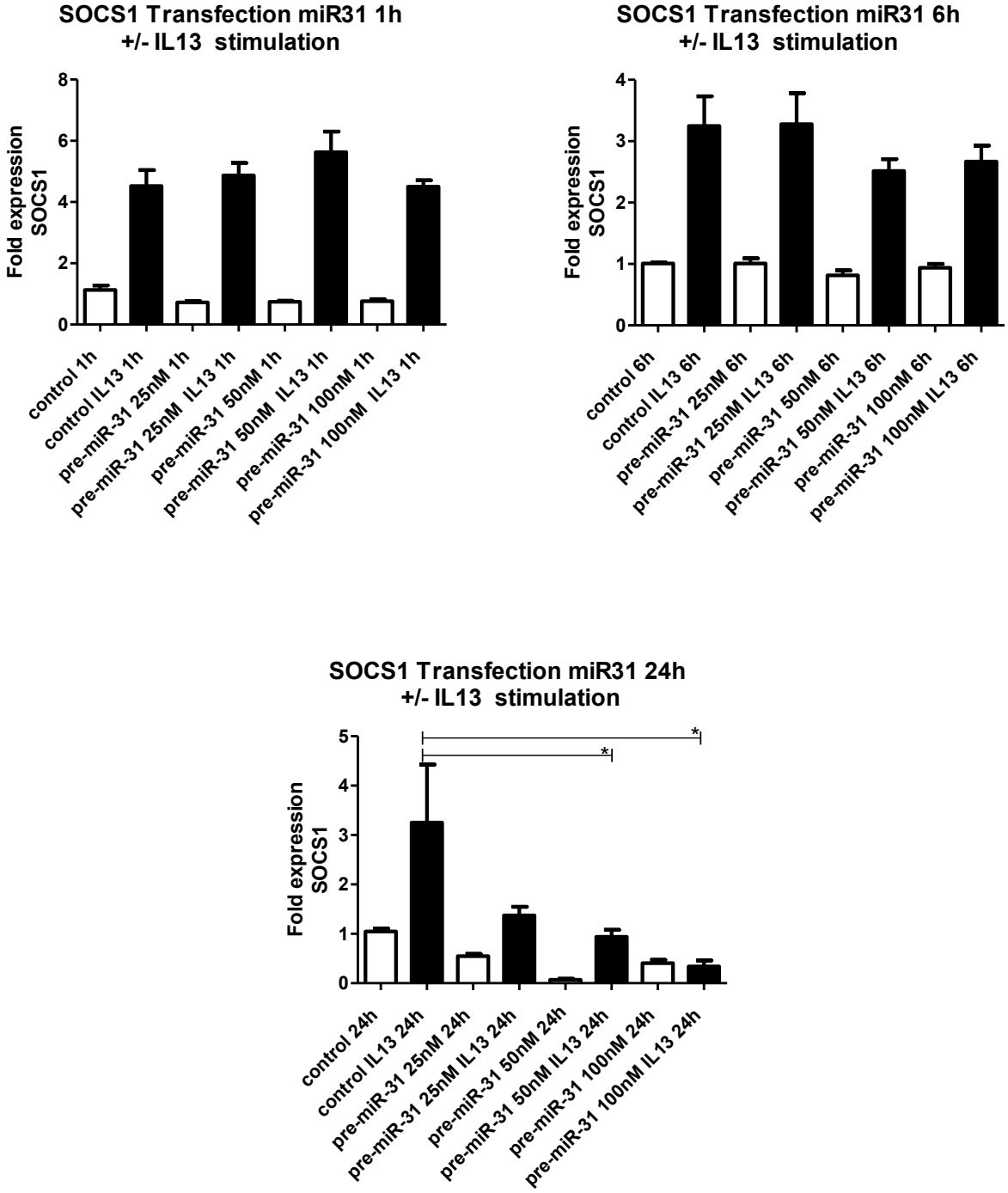


Figure 37 SOCS1 mRNA expression in HT-29 cells after miR-31 transfection and +/- IL-13 stimulation. Time points were taken at 1, 6 and 24 hours. SOCS1 mRNA expression to IL-13 was reduced 6h post transfection with 50nM and 100nM of miR-31 (no statistical significance). 24h post transfection IL-13-dependent response of SOCS1 expression was observed to be significantly down-regulated in the 2 higher doses of pre-miR-31transfection. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

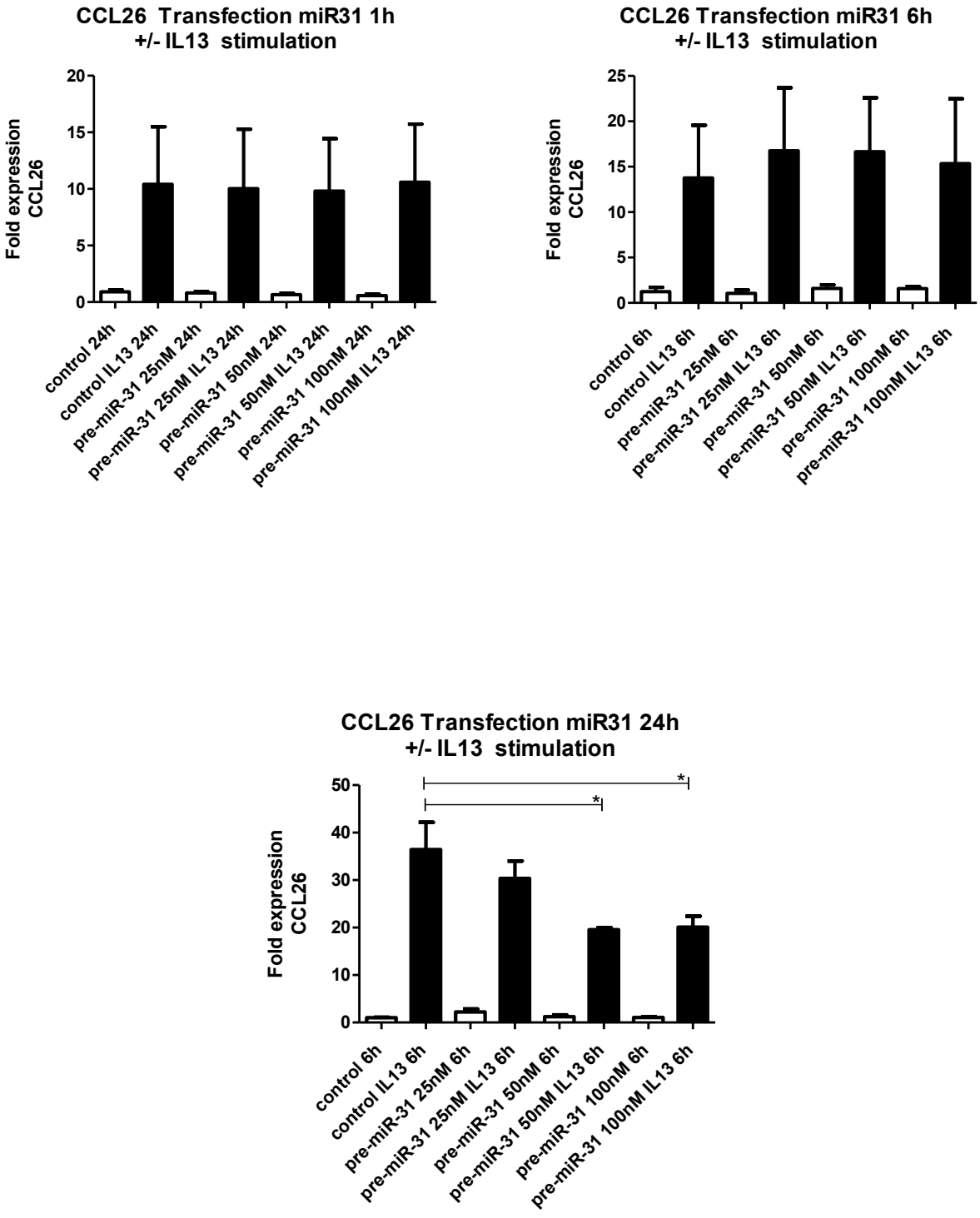


Figure 38. CCL26 mRNA expression in HT-29 cells after miR-31 transfection and +/- IL-13 stimulation. Transfected HT-29 cells with pre-miR-31 at 1, 6 and 24 hours were assayed for CCL26 mRNA expression. CCL26 mRNA levels peaked at 24 hours upon IL-13 stimulation. It was significantly reduced at 50 and 100nM pre-miR-31 post IL-13 stimulation. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

IL13Rα1 did show a highly significant down-regulation of its mRNA after 24 hours in keeping with previous transfection experiments with pre-miR-31. ***Figure 39*** demonstrates the reduction of IL13Rα1 in the course of time over 24 hours.

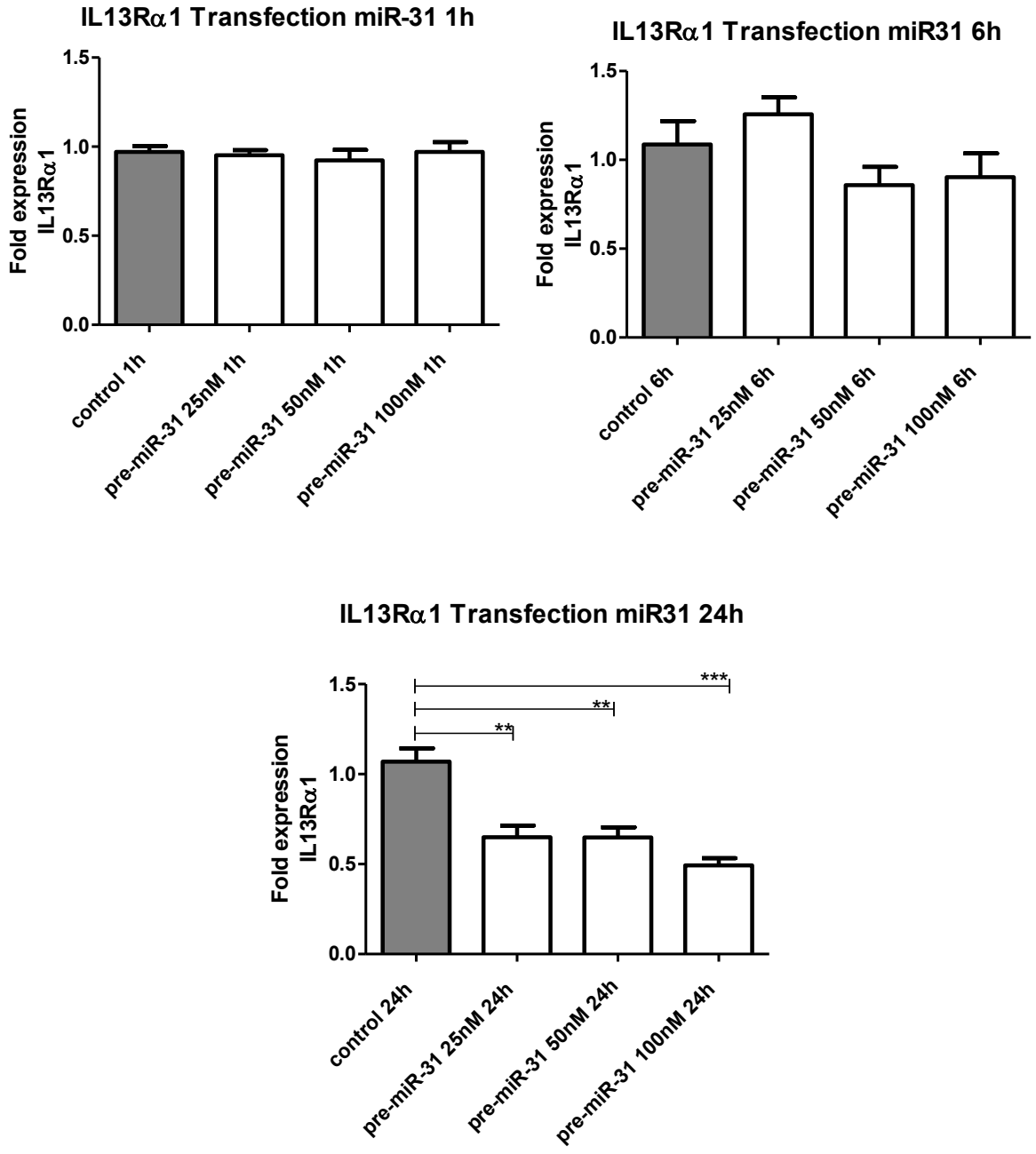


Figure 39. IL-13R1 mRNA expression in HT-29 cells after miR-31 transfection and +/- IL-13 stimulation. Transfected HT-29 cells with pre-miR-31 at 1, 6 and 24 hours showed a highly significant down-regulation of IL-13R1 mRNA after 24h. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

5.2.7.1 Transfection of HT-29 cells with pre-miR-31 and pre-miR-155 and their combination for 48hours +/- IL-13 stimulation

Following the encouraging results of the previous transfections of HT-29 cells with pre-miR-31 and pre-miR-155 and their combination showing a reduction in IL13Rα1 and a subsequent reduction of the IL-13 dependent genes SOCS1 and CCL26, we modified our transfection protocol (see ***section 2.11.1***) by prolonging the transfection time to 48 hours to achieve a more pronounced effect of the microRNAs on its target IL13Rα1.

Figure 40 shows the time course of IL13Rα1 mRNA expression in HT-29 cells at 1, 6 and 24 hours post IL-13 stimulation with 100ng/ml following transfection with pre-miR-31 and pre-miR-155 (100nM each) and the combination of the two microRNAs at 50nM each.

After 1 hour the expression of IL13Rα1 was already significantly reduced in the pre-miR-31 group and also in the combination of the 2 microRNAs. At 6 hours a statistically significant difference was seen in the combination group of pre-miR-31 and pre-miR-155, but the full effect of the reduction of IL13Rα1 was observed after 24 hours, when all transfected samples were significantly reduced in their mRNA expression of IL13Rα1, with the combination treatment of pre-miR-31 and pre-miR-155 achieving the strongest down-regulation of IL13Rα1 mRNA.

Figure 41 plots the changes in mRNA expression in the time course of SOCS1 at 1, 6 and 24 hours after transfection with pre-miR-31, pre-miR-155 and the combination of the 2 microRNAs.

At one hour all samples stimulated with IL-13 were reduced in expression compared with the control samples, with the pre-miR-31 and pre-miR-155 reaching significance.

At 6 hours mRNA expression of SOCS1 was significantly reduced in the IL-13 stimulated samples of pre-miR-31 and the combination of pre-miR-31 and pre-miR-155. Again as seen in the expression of the IL13Rα1 mRNA full effect of the transfection with our microRNAs and their combination was seen 24 hours post 48 hour transfection. SOCS1 was significantly reduced in all transfected groups diminishing the response to IL-13 stimulation to about 50 % compared to the control sample.

Figure 42 depicts the time course of CCL26 mRNA expression of the transfection model with pre-miR-31, pre-miR-155 and their combination after IL-13 stimulation at 1, 6 and 24 hours.

At 1 and 6 hours the IL-13 response of CCL26 mRNA expression was reduced in all transfected samples with the pre-miR-31 groups reaching significance. At 24 hours, at the peak of CCL26 expression, following IL-13 stimulation all transfected groups were highly significantly decreased, again confirming the peak of transfection efficiency to be at 24 hours after the 48 hours transfection.

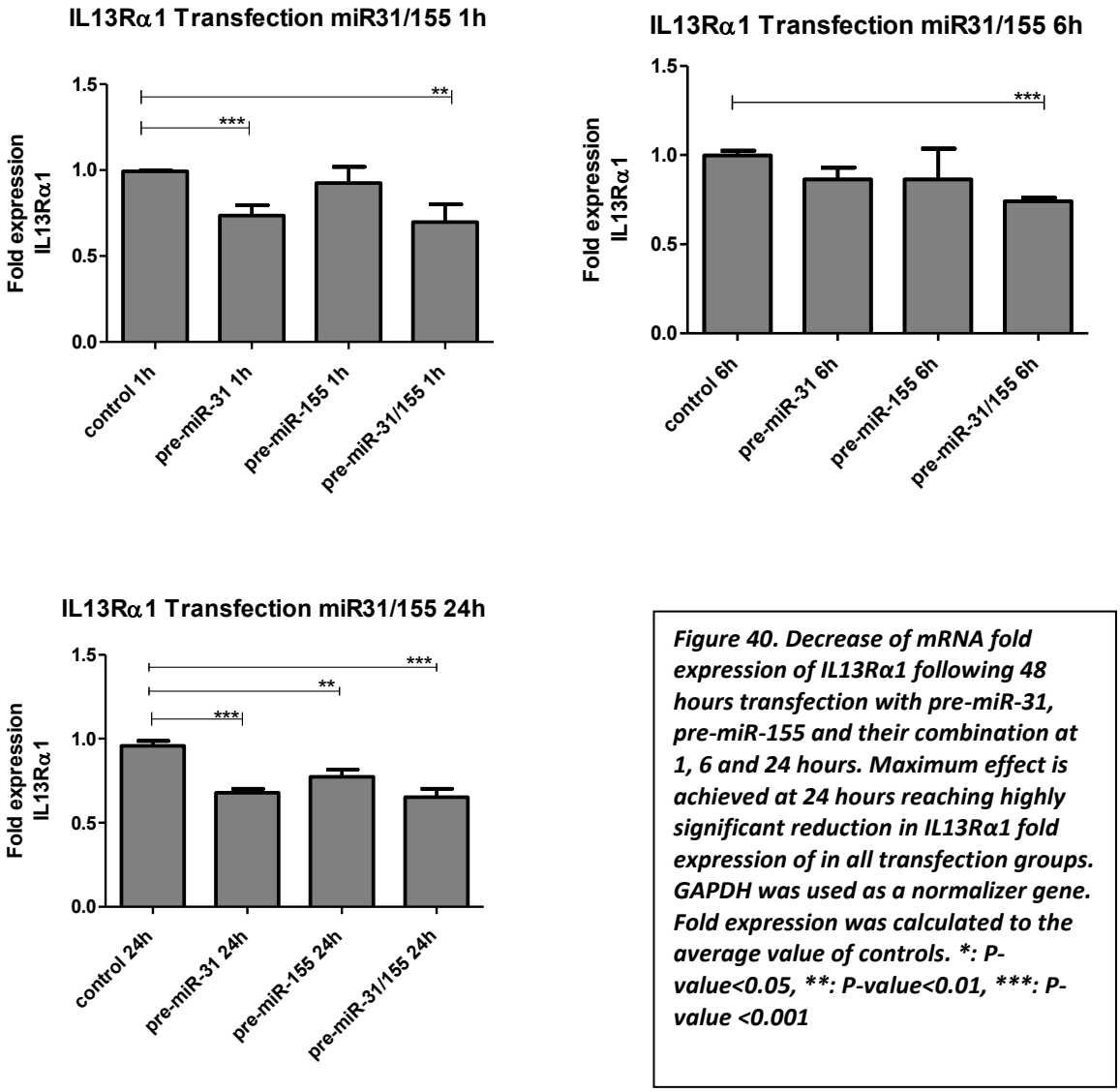
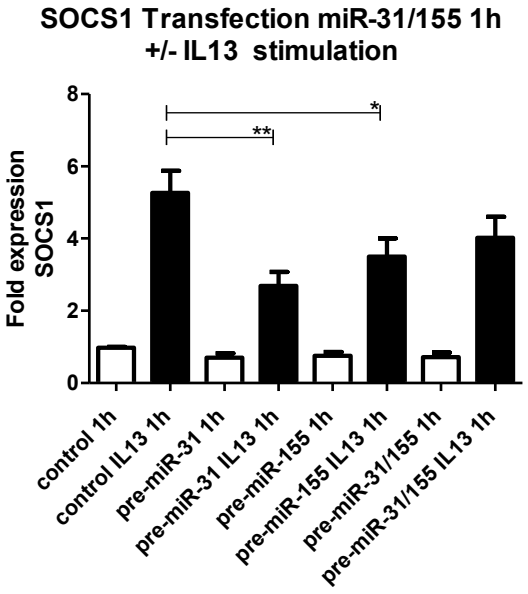
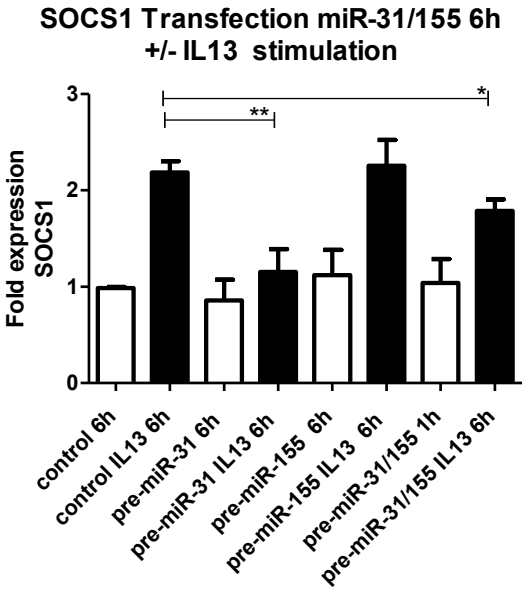


Figure 40. Decrease of mRNA fold expression of IL13Rα1 following 48 hours transfection with pre-miR-31, pre-miR-155 and their combination at 1, 6 and 24 hours. Maximum effect is achieved at 24 hours reaching highly significant reduction in IL13Rα1 fold expression of in all transfection groups. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001





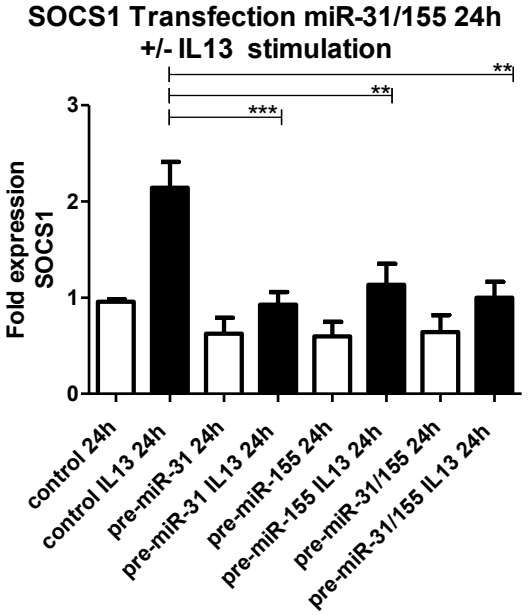
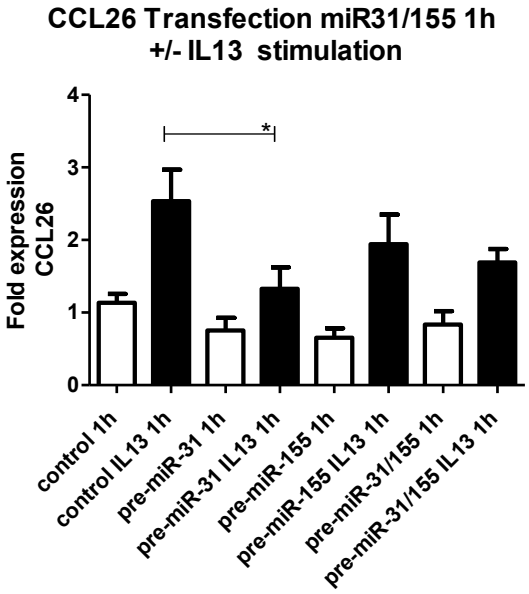
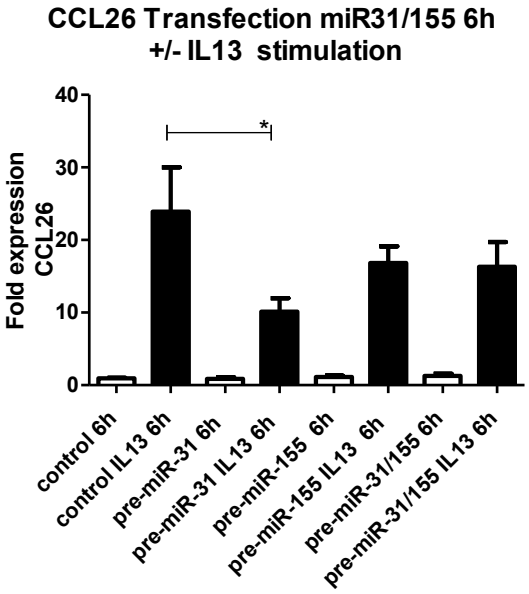


Figure 41. Decrease of mRNA fold expression of SOCS1 following 48 hours transfection with pre-miR-31, pre-miR-155 and their combination at 1, 6 and 24 hours. Maximum effect is achieved at 24 hours reaching highly significant reduction in fold expression of SOCS1 in all transfection groups. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001





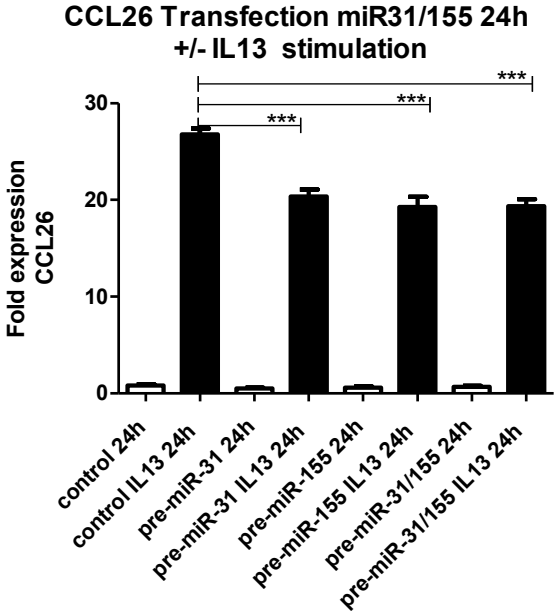


Figure 42. Decrease of mRNA fold expression of CCL26 following 48 hours transfection with pre-miR-31, pre-miR-155 and their combination at 1, 6 and 24 hours. Maximum effect is achieved at 24 hours reaching highly significant reduction in fold expression of CCL26 in all transfection groups. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

5.2.7.2 Protein expression of IL13Rα1 and IL-13 dependent phosphorylation of STAT6 in HT-29 cells treated with pre-mir-31, pre-miR-155 and in combination.

As previously described, IL-13 signalling through IL13Rα1 activating STAT6 plays an important role in the pathogenesis of UC, which has been highlighted by studies showing that ultimate down-regulation of the JAK-STAT6 pathway with siRNAs or HDAC inhibitors leads to a reduction of the toxic effects of IL-13 to epithelial cells (Rosen, Frey et al. 2011).

Transfections of HT-29 cells with pre-miR-31 and pre-miR-155 and their combination were undertaken in the same fashion as for the mRNA expression of IL13Rα1, SOCS1 and CCL26. Cells were transfected with pre-miR-31 and pre-miR-155 and their combination for 48 hours and protein was extracted to measure IL13Rα1. In order to measure phosphorylated STAT6, cells were stimulated IL-13 (100ng/ml) and harvested after 1 hour.

Figures 43 and ***44*** show a bar graph of three western blots for IL13Rα1 and phospho-STAT6, respectively, with a representative western blot underneath.

IL13Rα1 protein expression was significantly reduced in all groups transfected with microRNAs (see ***Figure 43***), confirming the earlier finding of down-regulation of the receptor on mRNA level.

Phosphorylated STAT6 was also shown to be significantly down-regulated in a progressive fashion demonstrating the biggest effect in the combination group of miR-31 and miR-155 (see ***Figure 44***).

These two results confirm the effect of pre-miR-31 and pre-miR-155 causing a down-regulation of the IL13Rα1 and a subsequent progressive decrease of the phosphorylation of STAT6 in HT29 cells, indicating a possible role for these 2 microRNAs in the regulation of the IL-13/STAT6 pathway.

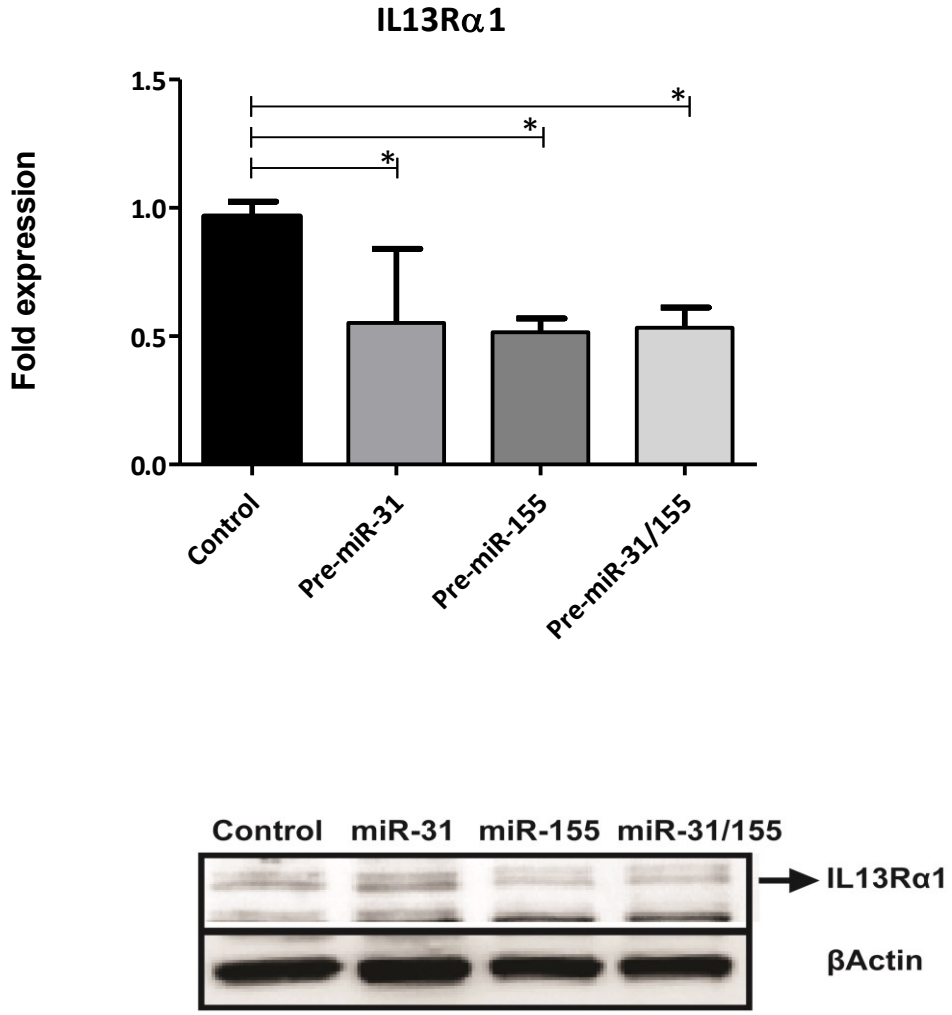


Figure 43. IL13Rα1 protein expression in pre-miR-31, pre-miR-155 and pre-miR-31/155 transfected HT-29 cells. IL13Rα1 protein expression is significantly reduced in all groups transfected with microRNAs, reaching its greatest suppression with the combination of miR-31 and miR-155. The diagram shows 3 western blots for IL13Rα1 with a representative western blot representing IL13Rα1 protein expression at the lower upper band. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

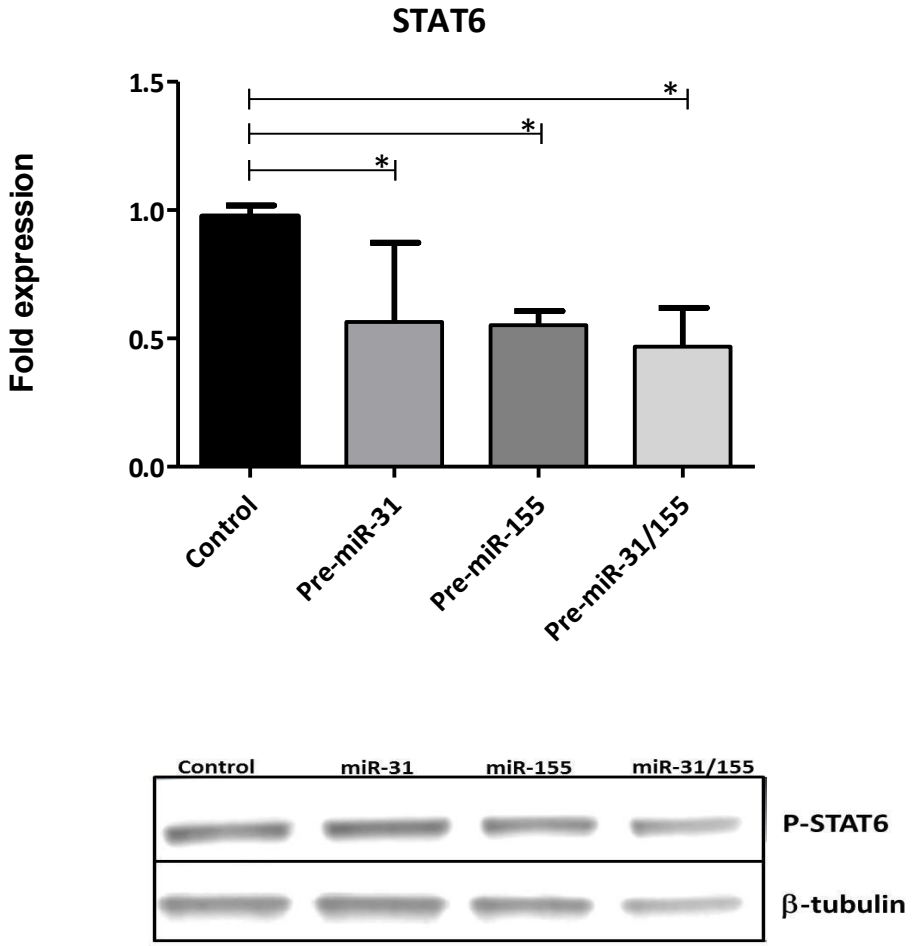


Figure 44. Phospho-STAT6 protein expression in pre-miR-31, pre-miR-155 and pre-miR-31/155 transfected HT-29 cells. Phosphorylation of STAT6 is significantly decreased in a progressive manner reaching its biggest effect in the combination group of pre-miR-31 and pre-miR-155. The diagram shows 3 western blots for phospho-STAT6 with a representative western blot. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

5.3 Functional role of miR-31 and miR-155 in an *in vitro* model representing lamina propria macrophages and monocytic cells (THP-1 cells)

5.3.1 Introduction

Biopsies of the colon provide samples consisting of mucosa and sub-mucosa and these two layers are affected by UC. To recreate this situation an *in vitro* model was set up with HT-29 cells, a human colonic epithelial cell line (see ***Chapter 6***). THP-1 cells, a monocytic cell line, were used to provide a model for macrophages and dendritic cells of the sub-mucosa. Experiments were set up to investigate the effect of miR-31 and miR-155 and their combination on the IL13Rα1 and on the IL-13 pathway in THP-1 cells.

As previously discussed, sampling of gut luminal antigens activates the innate mucosal immune response mainly facilitated by dendritic cells, macrophages and neutrophils mediated by enhanced Toll-like receptor (TLR) activity. This process determines as to whether an antigen is tolerated or whether action needs to be taken to eliminate it. This balance is disrupted in UC and leads to innocent antigens not being tolerated. This process stimulates dendritic cells and macrophages which initiate the differentiation of naïve T-cells into effector T-cells regulated by IL-10 and TGF-β. UC, a response predominately mediated through Th2 and NK T-cells producing IL-13, is initiated as a result of this loss of tolerance, leading to direct toxicity of IL-13 and NKT cells to gut epithelial cells and activation of the IL-13 and STAT6 pathway (Shih and Targan 2008, Matricon, Barnich et al. 2010, MacDonald, Monteleone et al. 2011, Strober and Fuss 2011).

THP-1 cells are a human leukemic cell line cultured from the blood of a boy with acute monocytic leukaemia (Tsuchiya, Yamabe et al. 1980). It provides a good *in vitro* model for monocytes, macrophages and dendritic cells. IL-13 signalling through IL13Rα1 activating STAT6 has previously been shown in THP-1 cells (Hart, Bonder et al. 1999).

The aim of the following experiments was to simulate the response of lamina propria macrophages and dendritic cells to stimulation with IL-13. In addition, we aimed to determine the effect of pre-miR-31 and pre-miR-155 and its combination on this cell type, to distinguish

whether there is a different response in the lamina propria as opposed to the epithelium. As mentioned above, UC affects both the epithelial layer and the lamina propria.

Firstly, the expression of IL-13 dependent genes found to be deregulated in our inflamed UC patient samples (***Figure 13***) was assessed in THP-1 cells. We found SOCS1, SERPINE1, CCL26 and IL13Rα1 to be expressed at CT values of 31, 29, 32 and 26 respectively in un-stimulated cells, but CCL18 and MMP9 were not sufficiently expressed.

5.3.2 IL-13 dose response experiment in THP-1 cells

Published data from Hebenstreit *et al* (Hebenstreit, Luft et al. 2003) suggests that IL-13 stimulation in epithelial cells produces a rise in SOCS1 with its peak of expression after one hour.

Following the previous problems with an ineffective IL-13 agent, new IL-13 was purchased (Recombinant human IL-13 (#213-IL-005, R&D Systems Europe Ltd., Oxfordshire, UK) and IL-13 stimulation experiments in THP-1 cells were set up using doses of 25, 50 and 100ng/ml. Cells were collected at 1, 6 and 24 hours to assess the expression response of SOCS1 and CCL26 mRNA by RT-qPCR (see ***Materials*** and ***Methods***).

Results revealed a slightly different result compared to that seen in HT-29 cells (***Figures 35 and 36***) although IL-13 stimulation showed that in general both SOCS1 and CCL26 expression was increased upon to IL-13 stimulation. SOCS1 mRNA expression showed a maximum response at 1 hour up to 20 fold at the maximum dose of IL-13 (100ng/ml). SOCS1 levels were still elevated 15 fold at 6 hours. At 24 hours SOCS1 was still 10 fold increased at the maximum dose of IL-13 (100ng/ml) (***Figure 45***).
The peak expression of CCL26 mRNA was 6 hours post-IL-3 stimulation showing a 10 fold increase, in contrast to the maximum rise of CCL26 in HT-29 cells at 24 hours (***Figure 46***). After 24 hours CCL26 was raised 3 fold at the maximum dose of 100ng/ml of IL-13.

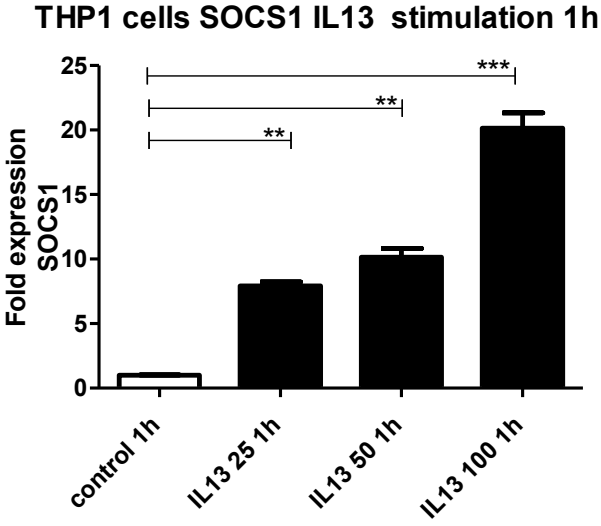
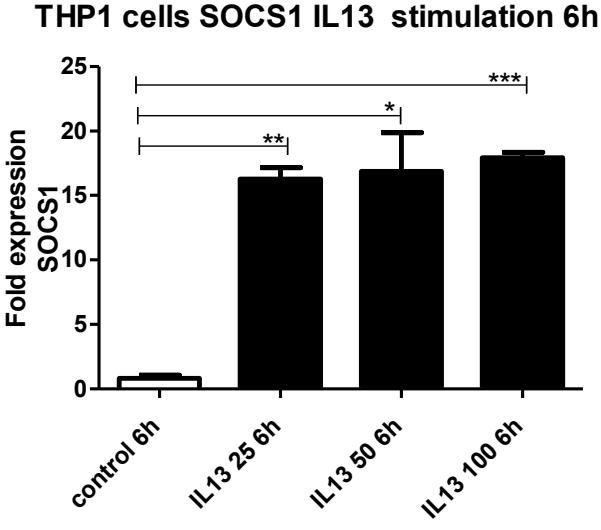
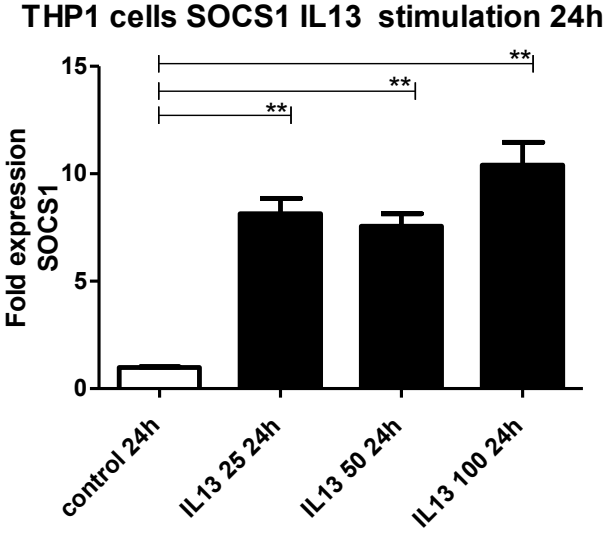


*Figure 45. SOCS1 mRNA levels in THP-1 cells upon IL-13 stimulation. SOCS1 mRNA levels were measured in THP-1 cells at 1, 6 and 24 hours after stimulation with 25, 50 and 100ng/ml of IL-13. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*





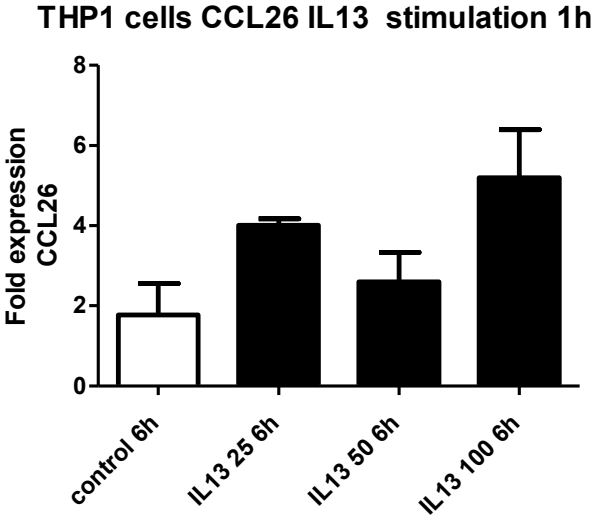
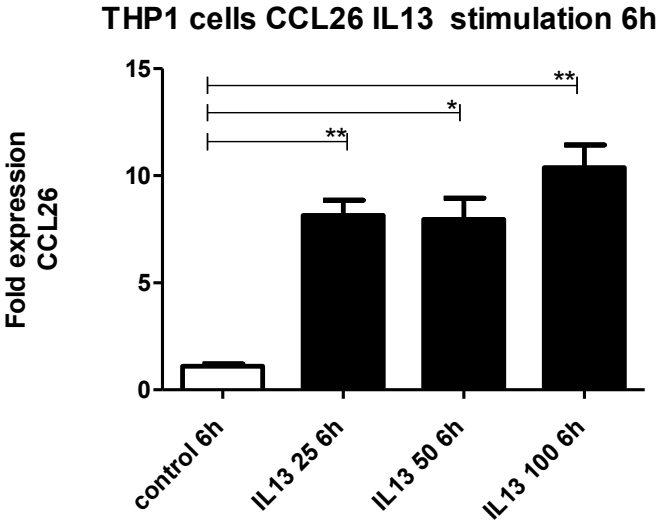
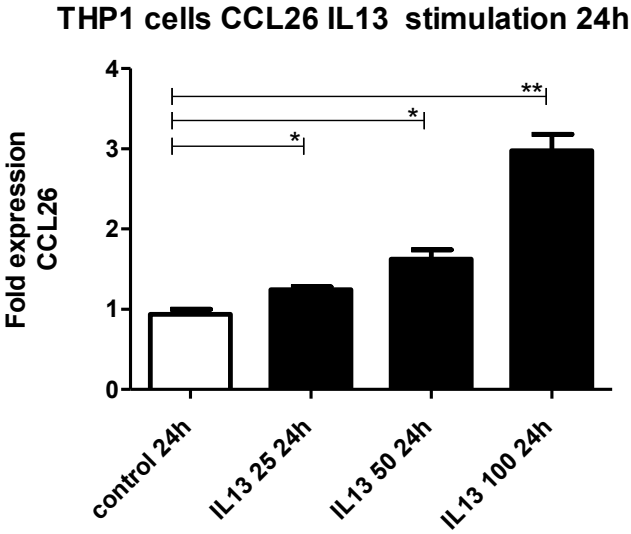


*Figure 46. CCL26 mRNA levels in THP-1 cells upon IL-13 stimulation. CCL26 mRNA levels were measured in THP-1 cells at 1, 6 and 24 hours after stimulation with 25, 50 and 100ng/ml of IL-13. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*





5.3.3 Transfection of THP-1 cells with pre-miR-31 and pre-miR-155 and its combination

Experiments were also set up to look at pre-miR-31 (100nM), pre-miR-155 (100nM) and their combination (50nM of pre-miR-31 and pre-miR-155 each) using the new IL-13 batch at 100ng/ml. Expression of mRNA of SOCS1, CCL26 and IL13Rα1 at 24 hours, post IL-13 stimulation following 48 hours of transfection with microRNAs, was measured by RT-qPCR (see ***Materials*** and ***Methods***).

Results showed significant down-regulation of SOCS1 at 24 hours in IL-13 stimulated THP1 cells when transfected with pre-miR-31, pre-miR-155 and their combination (see ***Figure 47***).

CCL26 response to IL-13 stimulation was also significantly mitigated in all pre-miR transfection groups (***Figure 48***). IL13Rα1 mRNA levels did show reduction of the expression in all transfected groups and reached significance in the pre-miR-31/155 combination samples. (***Figure 49***)

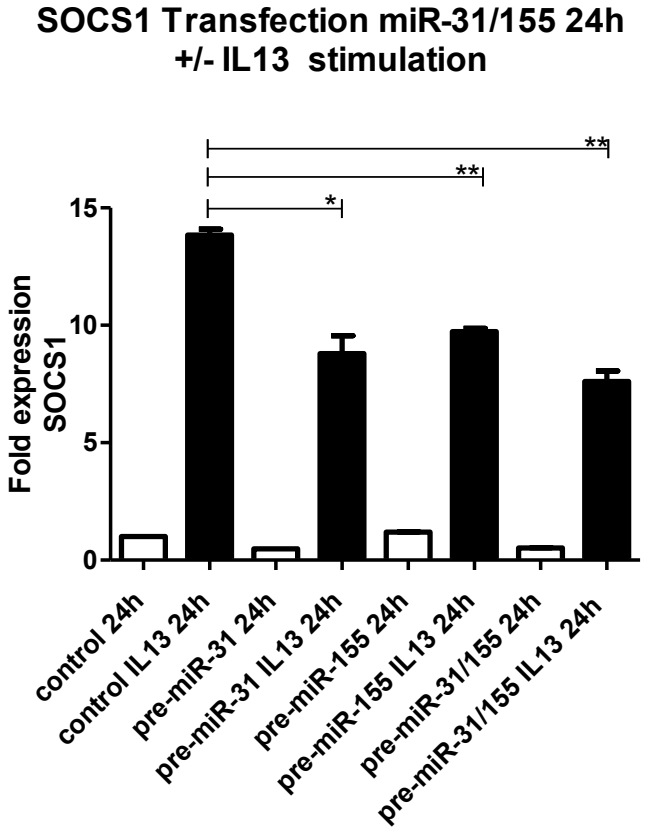


Figure 47. Effects of miR-31, miR-155 and miR-31/155 transfection in the IL-13 dependent expression of SOCS1 mRNA in THP-1 cells. THP-1 cells were transfected with pre-miR-31, pre-miR-155 and its combination. Cells were stimulated with IL-13 at 100ng/ml after 24 hours and harvested 48h post transfection. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

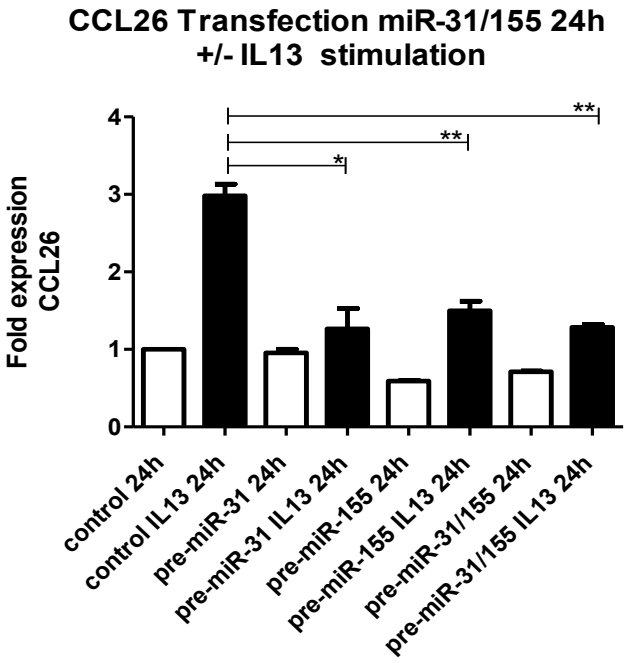


*Figure 48. Effect of miR-31, miR-155 and miR-31/155 transfection in the IL-13 dependent expression of CCL26 mRNA in THP-1 cells. THP-1 cells were transfected with pre-miR-31, pre-miR-155 and its combination. Cells were stimulated with IL-13 at 100ng/ml after 24 hours and harvested 48h post transfection. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*

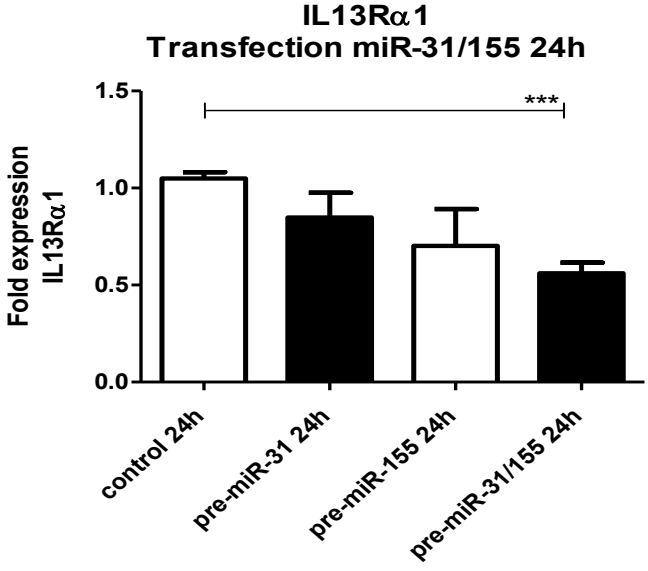


*Figure 49. Effects of miR-31, miR-155 and miR-31/155 transfection on the IL-13 dependent expression of IL13Rα1mRNA in THP-1 cells. mRNA expression of IL13Rα1was determined following transfection of THP-1 cells with pre-miR-31 and pre-miR-155 and its combination, stimulation with IL-13 at 100ng/ml at 24 hours and harvested at 48h post-transfection. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*

5.4 Functional role of miR-31 and miR-31 in human primary macrophages

5.4.1 Introduction

Following the encouraging, but inconsistent results in THP-1 cells confirming a general trend of down-regulation of IL13Rα1 after transfection with miR-31 and miR-155 leading to reduced expression of IL-13 dependent genes after stimulation with IL-13 similar to effects observed in epithelial cells, we wanted to confirm these results in human macrophages derived from peripheral blood monocytes. As transfection of THP-1 cells proved very challenging and did not always give consistent results, these experiments were set up to help create a better model for lamina propria mononuclear cells of the innate immune system.

As previously discussed, Martinez-Nunez *et al.* have identified miR-155 as a crucial regulator in human macrophages between the M1 (classically activated, pro-Th1) and M2 (alternatively activated, pro-Th2) responses through directly targeting IL13Rα1 and reducing levels of IL13Rα1 protein. This lead to diminished activation of STAT6 determining the M2 phenotype in macrophages. MiR-155 affected the IL-13-dependent regulation of several genes (SOCS1, DC-SIGN, CCL18, CD23, and SERPINE1) involved in the establishment of an M2/pro-Th(2) phenotype in macrophages (Martinez-Nunez, Louafi et al. 2011).

IL-13 seems not only to play an important role in the disruption of the epithelial barrier function (Heller, Fuss et al. 2002, Heller, Florian et al. 2005), but taking the results from THP-1 cells and macrophages as a model for the innate mononuclear cells in the lamina propria, a clear up-regulation of the IL-13/ STAT6 pathway can be observed. This could be influenced by microRNAs miR-31 and miR-155 targeting the IL13Rα1 in THP-1 cells, and it was previously shown that these microRNAs are up-regulated in our patient samples (***Figure 16***).

A reduction of IL13Rα1 following transfection with miR-155 in macrophages has previously been shown by our group (Martinez-Nunez, Louafi et al. 2011) and investigations concentrated on the effects of miR-31 and the combination of the two microRNAs directly targeting the IL13Rα1 in human macrophages.

5.4.2 Transfection of human macrophages with pre-miR-31 and pre-miR-155 and its combination

Monocyte derived human macrophages (obtained from healthy donors as in ***section 2.10.4***) were transfected with pre-miR-31 (100nM), pre-miR-155 (100nM) and its combination (50nM of pre-miR-31 and pre-miR-155 each) as in ***2.11.3***. After 48 hours of transfection cells were stimulated (or untreated) with IL-13 at 100ng/ml for another 24 hours. Expression of mRNA of CCL18, SOCS1 and IL13Rα1 after 24 hours was measured by RT-qPCR (see ***2.16, 2.17*** and ***2.18***).
Results showed a down-regulation of CCL18 at 24 hours in IL-13 stimulated human macrophages when transfected with pre-miR-31 and a significant mitigation of the IL-13 response with pre-miR-155. Combination of pre-miR-31 and pre-miR-155 showed the most pronounced effect and was highly significant (***Figure 50***).
SOCS1 response to IL-13 stimulation was also mitigated in all pre-miR transfection groups and reached significance in the pre-miR-155 and the combination group (***Figure 51***). IL13Rα1 mRNA levels did show reduction of the expression in all transfected groups and was highly significantly reduced in all pre-miR transfection groups (***Figure 52***).

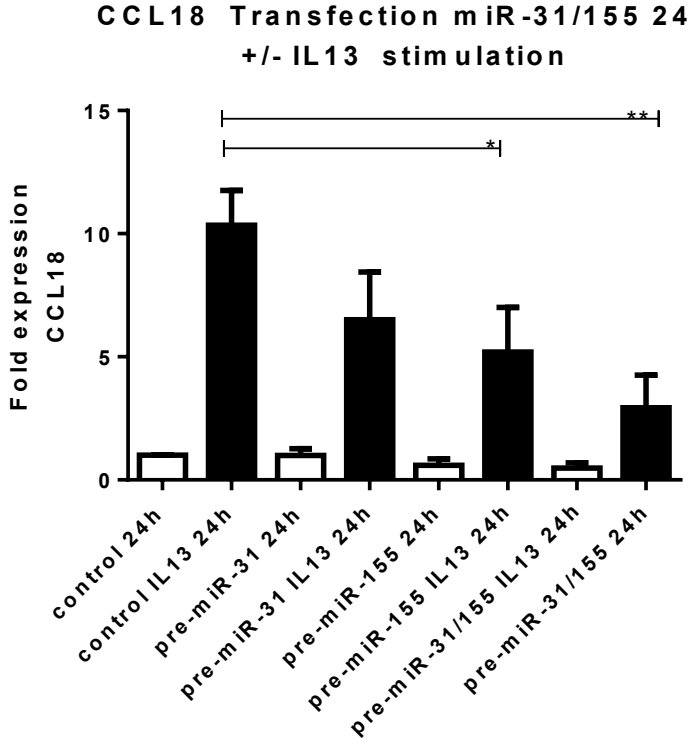


Figure 50. Effects of miR-31, miR-155 and miR-31/155 transfection in the IL-13 dependent expression of CCL18 in human primary macrophages. CCL18 mRNA expression was measured following transfection of human macrophages with pre-miR-31 and pre-miR-155 and its combination and stimulation with IL-13 at 100ng/ml at 24 hours. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

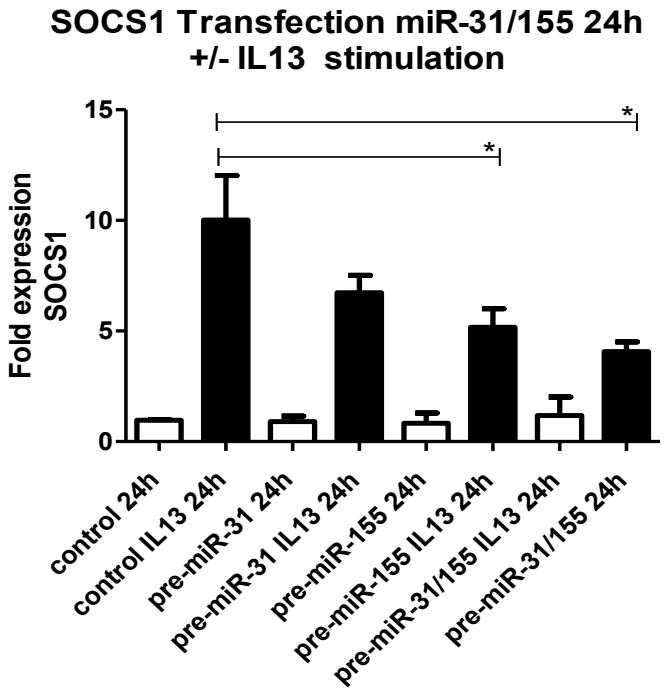


*Figure 51. Effects of miR-31, miR-155 and miR-31/155 transfection in the IL-13 dependent expression of SOCS1 in human primary macrophages. SOCS1 mRNA expression was measured following transfection of human macrophages with pre-miR-31 and pre-miR-155 and its combination, stimulation with IL-13 at 100ng/ml at 24 hours and collection after 24h stimulation. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*

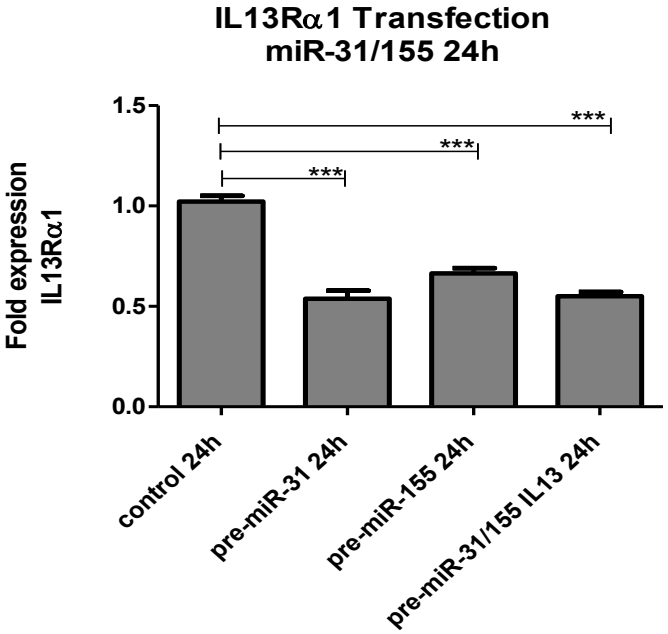


*Figure 52. mRNA expression of IL13Rα1 following transfection of human macrophages with pre-miR-31 and pre-miR-155 and its combination. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*

5.5 Discussion

Our *in vitro* experiments and cellular models aimed to dissect the role of miR-31 and miR-155 in the IL13Rα1 pathway in UC. Employing luciferase assays, we show that miR-31 directly binds to the 3'UTR of IL13Rα1 mapping its binding site to position 1146. This experiment also provides a direct molecular link in our previous findings in patient data showing a reduction in IL13Rα1 in active disease with high levels of miR-31. Together with miR-155 which directly targets IL13Rα1 (Martinez-Nunez, Louafi et al. 2011) our findings suggest a potential direct involvement of microRNA-31 in UC. MiR-31 and miR-155 could therefore play a significant role in the course of IBD influencing the activation of the JAK-STAT6 pathway and the direct toxic effect of IL-13 and NKT cells to the epithelium in patients with UC.

Both microRNAs miR-31 and miR-155 have been shown to be deregulated in UC (Fasseu, Treton et al. 2010, Takagi, Naito et al. 2010, Olaru, Selaru et al. 2011). IL-13, up-regulated in UC, alongside IL-4 induces activation of STAT6 via the IL13Rα1 (Fuss, Heller et al. 2004), which perpetuates the inflammatory cycle. UC is associated with increased colonic epithelial STAT6 phosphorylation, and STAT6 inhibition with STAT6 siRNA prevents IL-13-induced apoptosis and barrier disruption. Down-regulation of IL-13-induced STAT6 phosphorylation with SAHA, an HDAC-inhibitor, inhibited apoptosis and claudin-2 expression, and mitigated IL-13-induced reductions in trans-epithelial resistance in HT-29 cells, which makes STAT6 a possible therapeutic target in UC (Rosen, Frey et al. 2011).

As pointed out previously, miR-31 expression seems to change in a stepwise fashion as colonic tissue goes from normal to chronically inflamed to actively inflamed to neoplastic and could have a role in risk stratifying patients with long term IBD with regards to cancer risk (Olaru, Selaru et al. 2011). Therefore, miR-31 may well play an important role in the link between inflammation and cancer in UC.

TNF-α is of vital importance in the pathogenesis of UC and CD and has been shown to induce both miR-31 and miR-155 expression, as considered in previous discussions (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007, Suarez, Wang et al. 2010). TNF-α is a direct target in IBD therapy with anti-TNF therapy such as Infliximab and Adalimumab. Such biologic therapies may therefore have a profound influence on the expression of these microRNAs in IBD tissues.

The effect of drugs used in IBD therapy on the expression of microRNAs is unknown and will be discussed at a later point in this thesis (***Figures 60 and 71***).

All together these data suggest that there may be a potential role for using miR-31 and miR-155 as therapeutic option in UC with regards to possible anti-inflammatory and anti-neoplastic effects leading to a down-regulation of IL13Rα1 and preventing excessive activation of the IL-13/STAT6 pathway.

Following these findings of both microRNAs (miR-31 and miR-155) directly targeting the IL13Rα1, we decided to further investigate the role of these two microRNAs on the expression of IL13Rα1 and IL-13/STAT6 pathway employing *in vitro* experiments in different cellular models.

We firstly employed HT-29 cells, a colonic epithelial cell line. Following the testing of various genes of the IL-13 and TGF-β pathway in HT-29 cells and after the resolution of the ineffective IL-13 stimulation due to a presumably inactive IL-13 batch, we were able to show stimulation of the IL-13 dependent genes SOCS1 and CCL26 in HT-29 cells, previously shown to be significantly elevated in the paired active UC samples (***Figure 13***).

Results of the stimulation of IL-13-dependent SOCS1 expression were very similar to those published by Hebenstreit *et al*. (Hebenstreit, Luft et al. 2003). A maximum response of SOCS1 at 1 hour was shown, and increased expression of SOCS1 mRNA 6 fold at 1 hour at the maximum dose of 100ng/ml. SOCS1 was elevate 3 fold after 24 hours (***Figure 35***).

CCL26 has been chosen as candidate IL-13 dependent gene as previous IL-13 stimulation experiments have indicated that its mRNA expression increases after IL-13 treatment (***Figure 21***). CCL26 is expressed in HT-29 cells and was also elevated in our active UC group (***Figure 13***). In addition it also has been shown elevated in active UC (Manousou, Kolios et al. 2010). The peak expression of CCL26 was after 24 hours in contrast to the early rise of SOCS1. After 24 hours CCL26 was raised more than 50 fold at the maximum dose of 100ng/ml of IL-13 in HT-29 cells (***Figure 36***).

Transfection of gut epithelial HT-29 cells with pre-miR-31 shows that miR-31 clearly has an effect on IL13Rα1 reducing its expression to 40% compared to a control sample at a dose of 100nM (***Figure 29***) and this effects also the expression of the IL-13 dependent gene SOCS1

reducing its mRNA in a similar magnitude (***Figure 28***). The stimulation with IL-13 in these samples did not have the desired effect due to the old batch of IL-13 as pointed out above. Nevertheless there was a clear dose dependent effect on the expression of the IL13R α1 and SOCS1 with the maximum effect at 100nM of pre-miR-31.

No effect of pre-miR-31 transfection was seen on the expression of CCL5, CCL22 and SERPINE11 (see ***Figure 30***)

Pre-miR-155, targeting both SOCS1 and IL13Rα1, had a less dramatic effect reducing SOCS1 to about 70% and IL-13α1 by about 20% (see ***Figures 31*** and ***32***) when compared to the control. Again the stimulation with IL-13 in these samples did not have the desired effect due to the ineffective batch of IL-13 as pointed out above.

The effect of a combination of pre-miR-31 and pre-miR-155 at a concentration of 25nM each (a quarter of the original dose), on the mRNA expression of SOCS1 and IL13Rα1, reduced SOCS1 to about 50% compared to its control and IL13α1 by about 30% indicating a possible synergistic effect at this reduced dose (see ***Figures 33*** and ***Figure 34***). This was an interesting preliminary finding which could not be replicated in later experiments.

Repeating these experiments after using a new batch of IL-13 brought similar results as published by Hebenstreit *et al* (Hebenstreit, Luft et al. 2003) regarding the effect of the IL-13 stimulation on epithelial results and we were curious to see if the IL-13 induced stimulation of SOCS1 and CCL26 could be mitigated due to the reduction in the expression of IL13Rα1 observed in previous experiments following transfection with pre-miR-31 and pre-miR-155 and its combination.

These experiments were repeated at different time points at 1, 6 and 24 hours to see the true effect of the IL-13 stimulation with the new agent and its effect on SOCS1 and CCL26 mRNA expression, as well as on IL13Rα1 in the presence or absence of pre-miR-31 and pre-miR-155 and its combination. Repeating these experiments after using the new batch of IL-13 brought similar results as published by Hebenstreit *et al* (Hebenstreit, Luft et al. 2003) regarding the effect of the IL-13 stimulation.

Results of the repeat pre-miR-31 transfection showed that the response of SOCS1 to IL-13 could be reduced at 6 hours in the two higher doses of pre-miR-31 (50 and 100nM), but did not reach statistical significance. At 24 hours the IL-13 response of SOCS1 was significantly reduced at 50 and 100nM after transfection with pre-miR-31 mitigating the observed IL-13 induced mRNA expression of SOCS1 in the control sample (see ***Figure 37***).

CCL26 did not show a statistically significant response in the reduction of its mRNA expression after stimulation with IL-13 at 1 and 6 hours, but it peaked at 24 hours as demonstrated by the previous IL-13 stimulation (see ***Figure 36***). CCL26 was significantly reduced at the two highest doses of pre-miR-31 post IL-13 stimulation (see ***Figure 38***).
IL13Rα1 mRNA did show a statistically highly significant down-regulation after 24 hours in keeping with previous transfection experiments with pre-miR-31 in all the titrated doses with the maximum effect in the combination group of miR-31 and miR-155 (***Figure 39***).

Following the encouraging initial results of the previous transfections of HT-29 cells with pre-miR-31 and pre-miR-155 and their combination showing a reduction in IL13Rα1, we established a new transfection protocol, prolonging the transfection time to 48 hours, to achieve a more pronounced effect of the microRNAs on the down-regulation of its target IL13Rα1. Combination of the two microRNAs was dosed at 50nM each to be equal in dosing to single agents at 100nM.

IL13Rα1 was significantly reduced in the pre-miR-31 group and also in the combination of the 2 microRNAs just after 48 hours of transfection (corresponding with 1h IL-13 stimulation). At 6 hours a significant result is seen in the combination group of pre-miR-31 and pre-miR-155, but the full effect of the reduction of IL13Rα1 is observed after 24 hours, when all transfected samples are significantly reduced in their mRNA expression of IL13Rα1, with the combination treatment of pre-miR-31 and pre-miR-155 achieving the strongest down-regulation of IL13Rα1 mRNA (***Figure 40***).

The IL-13 dependent genes tested, SOCS1 and CCL26, showed reduction of expression in most microRNA treated groups at 1 and 6 hours post IL-13 stimulation (***Figures 41*** and ***42***, respectively). However the full effect was seen after 24 hours when all groups show a highly significant mitigation of the IL-13 response of these genes which are corresponding with the significant reduction of IL13Rα1 in these same samples.

This is a clear indication of the effects of pre-miR-31 and pre-miR-155 and its combination to affect the down-stream IL-13 pathway due to its effect on the down-regulation of IL13Rα1.

We have previously shown that IL13Rα1 is down-regulated in samples of UC that are actively inflamed at messenger RNA level and protein level (***Figures 13*** and ***14***-***15***, respectively) corresponding with high levels of miR-31 and miR-155 in these samples (***Figure 16***).

To prove that the observed down-regulation of the IL13Rα1 not only happens on messenger RNA level but also translates into reduced protein expression of the receptor, we performed western blotting of the IL13Rα1 in the same conditions in our HT-29 epithelial cell model. Furthermore, we also looked at phosphorylation levels of STAT6 to see if there was a decrease in STAT6 activation, due to decreased IL13Rα1 availability. As discussed before, Rosen et al have shown that ultimate down-regulation of the JAK-STAT6 pathway with siRNAs or HDAC inhibitors leads to a reduction of the toxic effects of IL-13 to epithelial cells (Rosen, Frey et al. 2011).

It was demonstrated that IL13Rα1 protein expression was significantly reduced in all groups transfected with microRNAs (***Figure 43***), confirming the earlier finding of down-regulation of the receptor on mRNA level (***Figure 40***).

This also corresponds with the observation made in colonic biopsy samples where we were able to show a significant reduction of IL13R α1 in the active UC group in mRNA (***Figure 13***) and protein expression (see ***Figures 14*** and ***15***) with corresponding high levels of miR-31 and miR-155 (***Figure 16***) measured, leading to the reduction of the receptor by directly targeting it (see ***Figure 20*** and (Martinez-Nunez, Louafi et al. 2011).

Very similar results were observed in epithelial cells isolated (see ***2.15***) from patients with unaffected inactive and active UC compared to normal controls. MiR-31 and miR-155 were significantly elevated in active UC (***Figure 18***) with corresponding significantly reduced expression of IL13Rα1 mRNA (***Figure 17***).

Phosphorylation of STAT6 was also significantly decreased gradually (see ***Figure 44***) reaching its biggest effect in the combination group of pre-miR-31 and pre-miR-155.

These two results confirm the effect of pre-miR-31 and pre-miR-155 causing a down-regulation of the IL13R α1 indicating a possible role for these 2 microRNAs exhibiting an anti-inflammatory influence.

Expression of miR-31 and miR-155 was significantly increased in our inflamed UC samples in whole biopsies and epithelial cell isolates (***Figures 16*** and ***18***), which confirms the findings of previous studies conducted showing an increase in miR-31 and miR-155 in inflamed UC tissue (Fasseu, Treton et al. 2010, Takagi, Naito et al. 2010, Olaru, Selaru et al. 2011).

SOCS1 was also significantly raised in the inflamed contingent of our UC samples (***Figure 13***) and epithelial cell isolates (***Figure 17***) alongside with other IL-13 dependent genes such as CCL18, SERPINE1 and MMP9 in active UC biopsies (***Figure 13***), indicating an IL-13 driven response typical of UC through the activation of the JAK-STAT6 pathway and the direct toxic effect of IL-13 and NKT cells to the epithelium.

IL13Rα1 mRNA was reduced in our inflamed UC tissue samples from biopsies (***Figure 13***) and epithelial cell isolates (***Figure 17***) and was also shown to be significantly decreased in its protein expression in active UC colonic biopsies as compared to normal samples and unaffected inactive UC (***Figures 14*** and ***15***).

The *in vitro* experiments with pre-miR-31 and pre-miR-155 and a combination of the two microRNAs on HT-29 epithelial cells clearly displayed a significant effect on the expression of SOCS1, CCL26 and IL13Rα1 expression. The response to IL-13 of SOCS1 and CCL26 mRNA was clearly reduced and may even be more pronounced after a longer transfection period. There was no clear evidence of a possible synergistic effect of miR-31 and miR-155 in these experiments with HT29 cells.

Each individual microRNA (miR-31 and miR-155) and the combination of the two, influence the activation of IL-13 dependent genes by down-regulating the IL13Rα1 on mRNA (see ***Figures 40, 41*** and ***42***) and protein level (see ***Figure 43***) leading to a reduced activation of the JAK-STAT6 pathway, as demonstrated by the progressive reduction in phosphorylated STAT6 (see ***Figure 44***).

The high levels of miR-31 and miR-155 in inflamed UC tissue causing a down-regulation of IL13Rα1 may well be an attempt to stop the activation of the STAT6 pathway. High levels of SOCS1, an inhibitor of STAT6 activation, might reflect the first line of defence following increased IL-13 activation through IL13Rα1, especially considering its activation profile reaching the maximum expression at 1h (Hebenstreit, Luft et al. 2003). Reducing the expression of

IL13Rα1 employing microRNAs targeting this receptor seems a more long-term strategy to prevent tissue damage.

As previously described, IL-13 signalling through IL13Rα1 activating STAT6 plays an important role in the pathogenesis of UC. This has been highlighted by studies showing that ultimate down-regulation of the JAK-STAT6 pathway with siRNAs or HDAC inhibitors leads to a reduction of the toxic effects of IL-13 to epithelial cells (Rosen, Frey et al. 2011). It has also been shown that Interferon-β-1a, located in a chromosome cluster next to miR-31 gene (***Figure 4***), down-regulates STAT6 via activation SOCS1, another IL-13 regulated gene to achieve clinical remission in a subset of patients with active UC (Mannon, Hornung et al. 2011).

MicroRNAs targeting IL13Rα1 might well be able to reduce IL-13 induced JAK-STAT6 activation via down-regulation of IL13Rα1 in vivo and therefore influence the severity of inflammation similar to the studies describing down-regulation of STAT6 via siRNA, HDAC inhibitors and Interferon-β-1a (Rosen, Frey et al. 2011), (Mannon, Hornung et al. 2011)

After dissecting the role of miR-31 and miR-155 in epithelial cells we assayed their possible role in monocytes and macrophages, given their essential role in the immune response and immune regulation.

THP-1 cells were used to simulate the response of lamina propria macrophages and dendritic cells, vitally important in the innate immune response in UC. Investigations focused the effects of stimulation with IL-13 and transfection with pre-miR-31 and pre-miR-155 and their combination on this cell type to assay the consequences these microRNAs have on the expression of IL-13 dependent genes SOCS1, CCL26 and IL13Rα1. Our aim was to develop a potential model for lamina propria macrophages and dendritic cells and to investigate potential differences in the response compared to epithelial cells, given that UC affects both the epithelial layer and the lamina propria.

Results with THP-1 cells have been similar but less conclusive as compared to HT-29 epithelial cells (see ***Chapter 8***) and generally results were less consistent and much more difficult to reproduce. Experiments of IL-13 stimulation (with new IL-13 reagent) showed consistent results in terms stimulation efficiency for SOCS1 and CCL26 (see ***Figure 45*** and ***46***, respectively).

Results demonstrated a significant down-regulation of SOCS1 at 24 hours in IL-13 stimulated THP1 cells when transfected with pre-miR-31, pre-miR-155 and their combination (see ***Figure 47***). CCL26 response to IL-13 stimulation was also significantly mitigated in all pre-miR transfection groups (***Figure 48***). IL13Rα1 mRNA levels did show reduction of the expression in all transfected groups and reached significance in the pre-miR-31/155 combination samples. (***Figure 49***)

IL13Rα1 mRNA expression was down-regulated by both pre-miR-31 and pre-miR-155 and significantly reduced by their combination, which was translated into significant reductions in all pre-miR transfection groups of SOCS1 and CCL26 (see ***Figures 47, 48*** and ***49***).

Generally transfection of THP-1 cells proved very challenging due to the inconsistency of results, which may reflect variable transfection efficiency of THP-1 cells and other members of our group have experienced similar problems in the past.

Therefore, experiments in THP-1 cells mainly confirmed results observed in HT-29 cells (colonic epithelial cells). However, THP-1 cells did not prove an ideal model for lamina propria mononuclear cells of the innate immune system. Due to their inconsistency we decided to repeat these experiments with human blood derived macrophages from healthy donors.

Experiments in monocyte derived human macrophages confirmed the findings observed in THP-1 cells and both together were used as model for mononuclear cells of lamina propria, such as macrophages and dendritic cells, vital players in the innate immune response in UC. We investigated the effects of transfection with pre-miR-31 and pre-miR-155 and a combination of the two and stimulation with IL-13 on these human cells to assay the consequences these microRNAs have on the expression of IL-13 dependent genes CCL18, SOCS1, and IL13Rα1.

This second model for lamina propria macrophages and dendritic cells (see ***Chapter 7***) was set up to investigate potential differences in the response compared to epithelial cells, given that UC affects both the epithelial layer and the lamina propria.

Results with monocyte derived human macrophages from healthy donors have been similar to results in THP-1 cells (***Chapter 7***) and generally display the same effects of mir-31 and miR-155 and its combination that was observed in HT-29 epithelial cells (***Chapter 6***).

IL13Rα1 mRNA expression was significantly down-regulated by both pre-miR-31 and pre-miR-155 and by its combination (***Figure 52***), which was translated into reductions in all pre-miR transfection groups of CCL18 and SOCS1, most of which were significant (***Figure 50*** and ***51***, respectively).

Increased levels of CCL18 in active UC have been first described in this study (***Figure 13***). Produced by mononuclear cells, it is known to induce expression of chemokines attracting lymphocytes, dendritic cells and monocytes to the site of inflammation (Schutyser, Richmond et al. 2005). Being regulated by Th2 cytokines IL-4 and IL-13 and IL-10 (Meau-Petit, Tasseau et al. 2010), it was unsurprising that it was raised in active UC.

Targeting IL-13 signalling with microRNAs miR-31 and miR-155 reducing the IL13Rα1 achieves a significant down-regulation of the pro-inflammatory chemokine CCL18 (***Figure 50***).

We have now shown down-regulation of IL13Rα1 by miR-31 and miR-155 and its combination in in vitro experiments in epithelial cells, THP-1 cells and monocyte derived human macrophages, being able to significantly diminish the response to IL-13 dependent gene expression of SOCS1 (all cell types), CCL26 (epithelial cells) and CCL18 human macrophages. All these IL-13 dependent genes were significant elevated in our active UC biopsy despite the significantly reduced IL13Rα1 in active UC biopsies and UC epithelial cell isolates. In vivo significantly raised miR-31 and miR-155 seem to be a mechanism to protect excessive Il-13/STAT6 activation, but the system seems to get overwhelmed in severe inflammation.

Therefore we will investigate whether additional miR-31 and miR-155 can achieve further down-regulation of the IL13Rα1, achieving a similar result as observed in our 3 in vitro models.
To achieve this we will use an explant culture model with active UC colonic biopsies transfected with pre-miR-31 and pre-miR-155 and its combination.

6 Results 3: Transfection of active UC explant cultures with pre-miR-31, pre-miR155 and pre-miR-31/155 combination

6.1 Introduction

As discussed before, disruption of the mucosal barrier by the direct toxic effects of IL-13 and the presence of its main producer NKT-cells are the hallmarks of UC. It leads to increased mucosal permeability as first shown in murine studies (Heller, Fuss et al. 2002). IL-13 signalling through IL13Rα1 activating the STAT6/IL-13 pathways plays also an important role in the pathogenesis of UC leading to increased Th2 activation and perpetuation of inflammation. Down-regulation of the JAK-STAT6 pathway with siRNAs or HDAC inhibitors has been shown to lead to a reduction of the toxic effects of IL-13 to epithelial cells (Rosen, Frey et al. 2011).

Expression of miR-31 and miR-155 was significantly increased in our inflamed UC samples (***Figure 16***), which confirms the findings of previous studies conducted showing an increase in miR-31 and miR-155 in inflamed UC tissue (Fasseu, Treton et al. 2010, Takagi, Naito et al. 2010, Olaru, Selaru et al. 2011).

In the initial biopsy samples from normal colonic tissue, paired unaffected inactive and active UC patients, we were able to determine the increased expression of the genes of the IL-13 pathway along with a down-regulation of IL13Rα1 on mRNA and protein expression (***Figure 13*** and ***Figures 14*** and ***15***).

SOCS1 was significantly raised in the inflamed contingent of the UC samples alongside with other IL-13 dependent genes such as CCL18, CCL26, SERPINE1 and MMP9 (***Figure 13***), indicating an IL-13 driven response typical of UC through the activation of the JAK-STAT6 pathway and the direct toxic effect of IL-13 and NKT cells to the epithelium, despite the down-regulation of IL13Rα1 (mRNA and protein) in our inflamed UC tissue samples (***Figure 13*** and ***Figures 14*** and ***15***).

We interpreted these findings as an attempt by these 2 microRNAs (miR-31/miR-155), which target the IL13Rα1 directly (Martinez-Nunez, Louafi et al. 2011), to down-regulate the IL13Rα1

in order to break the cycle of the STAT6/IL13 pathway activation. In case of severe active UC we hypothesise that the system gets overwhelmed and therefore there is still significant up-regulation of IL-13 dependent genes and phosphorylation of STAT6 despite the down-regulation of IL13Rα1.

We were able to demonstrate that the expression of IL13Rα1 (both mRNA and protein) and CCL18, SOCS1 and CCL26 mRNAs as well as the IL-13 dependent phosphorylation of STAT6 can be significantly down-regulated in all of our *in vitro* models of colonic epithelial cells (HT-29 cells) (***Figures 40, 41, 42, 43*** and ***44***).

Similar results of a significant decrease of the in mRNA levels of IL131Rα1 were observed in in vitro models representing cells of the innate immune system after transfection with miR-31 and miR-155 and its combination. Human monocytes from healthy donors as well as THP-1 cells (even if less consistently), transfected by pre-miR-31 and pre-miR-155 and its combination demonstrated that these microRNAs mitigate the response of IL-13 dependent genes in the transfected groups as compared to controls following the down-regulation of the IL13Rα1 (***Figures 47, 48, 49, 50, 51,*** and ***52***).

Taken together, our data opens the possibility that modulation of microRNAs targeting IL13Rα1 could achieve a therapeutic benefit for patients with active UC.

The ultimate goal of this project is to show that transfection with miR-31 and miR-155 or their combination can achieve down-regulation of IL-13 dependent gene expression in human inflamed UC tissue. Our *in vitro* data employing HT-29 cells (our colonic epithelial cell model) and THP-1 cells and human monocytes (our lamina propria macrophage and dendritic cell model) have shown encouraging results so far and we hope to translate these effects on the down-regulation of IL13Rα1, SOCS1, CCL18 and CCL26 by miR-31 and miR-155 and its combination into patient samples.

The proposed experiments aim to achieve our main goal: assessing the deregulated IL-13 dependent mRNA changes seen in our patient data at the start of this project and establishing a potential therapeutic use of microRNAs miR-31 and miR-155 in active UC.

6.2 Transfection of active UC explant culture samples with miR-31 and miR-31 and their combination decreases mRNA levels of IL-13 dependent genes due to reduction of IL13Rα1

Fifteen patients with active UC (left sided UC or pan-ulcerative colitis) were initially recruited. For explant culture experiments UC samples from active UC (endoscopic Mayo score 2-3) were taken from the sigmoid colon of each patient. 8 patient data was excluded from the final analysis due to unacceptable GAPDH normalising gene CT values due to degradation of RNA following the 24 hours explant culture.

Demographic data was recorded including age, sex, diagnosis, duration and extent of disease (***Table 10***). Current drug history was carefully noted. In all patients with UC a full clinical and endoscopic Mayo score (***Table 4***) was calculated and is summarised in (***Table 10***).

Results revealed a significant down-regulation of IL13Rα1 with miR-31 and miR-155 at a dose of 100nM. The combination of the 2 microRNAs at 50nM each achieved a highly significant decrease of IL13Rα1 mRNA, achieving a further decrease of IL13Rα1 mRNA as compared to the two single doses (see ***Figure 53***).

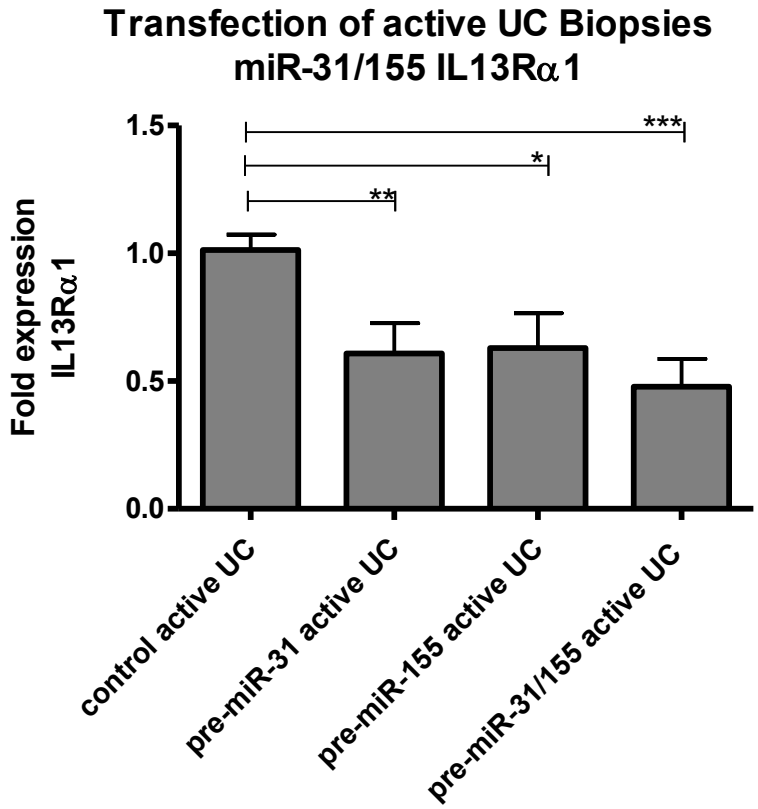
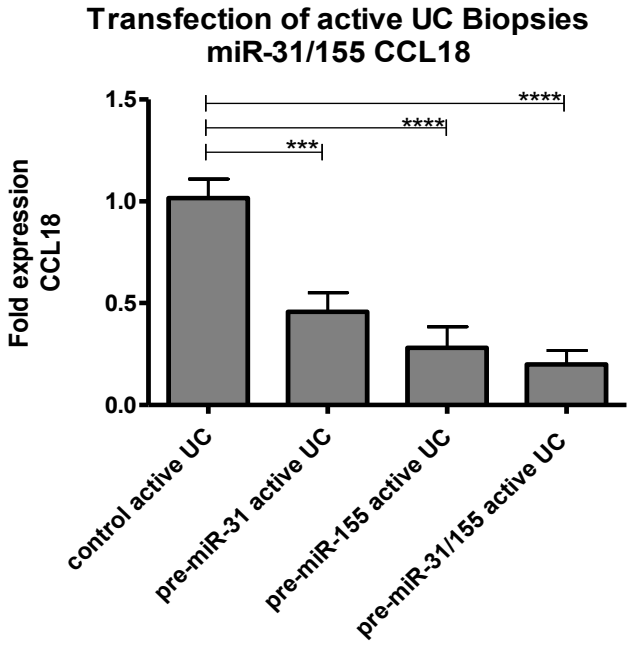


Figure 53. Down-regulation of IL13Rα1 mRNA in active UC explant cultures following transfection with microRNAs miR-31 and miR-155. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

A corresponding decrease in keeping with the reduction of IL13Rα1 was observed in the IL-13 dependent genes CCL18 and SOCS1 (see ***Figure 54***).

CCL18 was highly significantly reduced with miR-31, miR-155 and the combination of the microRNAs achieved the biggest decrease of CCL18 mRNA with a p-value of < 0.0001 (***Figure 54***).

SOCS1 was reduced significantly in the explant culture biopsies after transfection with pre-miR-31 and pre-miR-155 after 24 hours, but again the most pronounced effect was achieved with the combination of the 2 microRNAs, revealing a highly significant result (***Figure 54***).



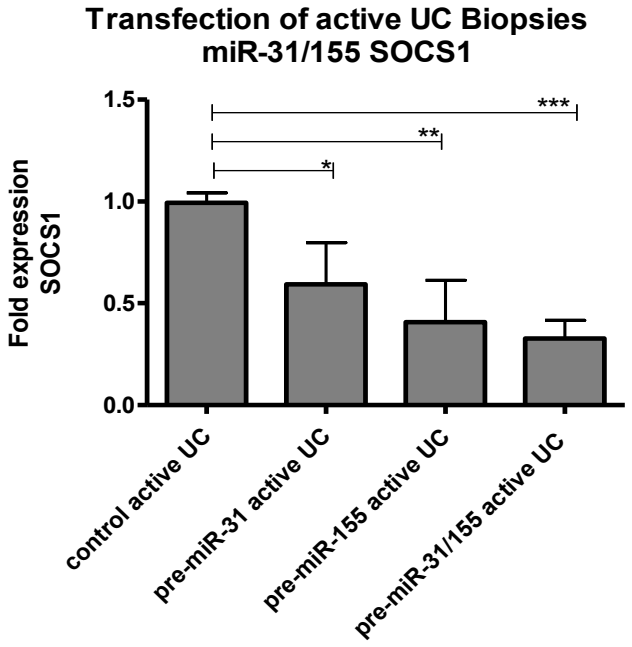


*Figure 54. Down-regulation of CCL18 and SOCS1 mRNA in active UC explant cultures following transfection with miR-31 and miR-155. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001*

No clinically significant modulation in the mRNA levels of SERPINE1 and CCL26 (***Figure 55)*** was seen in our active UC explant cultures, which could be explained by the presence of TGF-beta which also stimulates these genes apart from IL-13. We have shown the effects of TGF-beta stimulation on SERPINE1 and CCL26 in epithelial HT-29 cells (***Figure 22***).

MMP9 was seen to be increased in all microRNA treatment groups and may just generally represent higher levels of MMP9 due to tissue degradation releasing increased levels of MMP9 (***Figure 55***).

No significant differences were seen in the expression of TNF-α as compared to the control group (see ***Figure 55***).

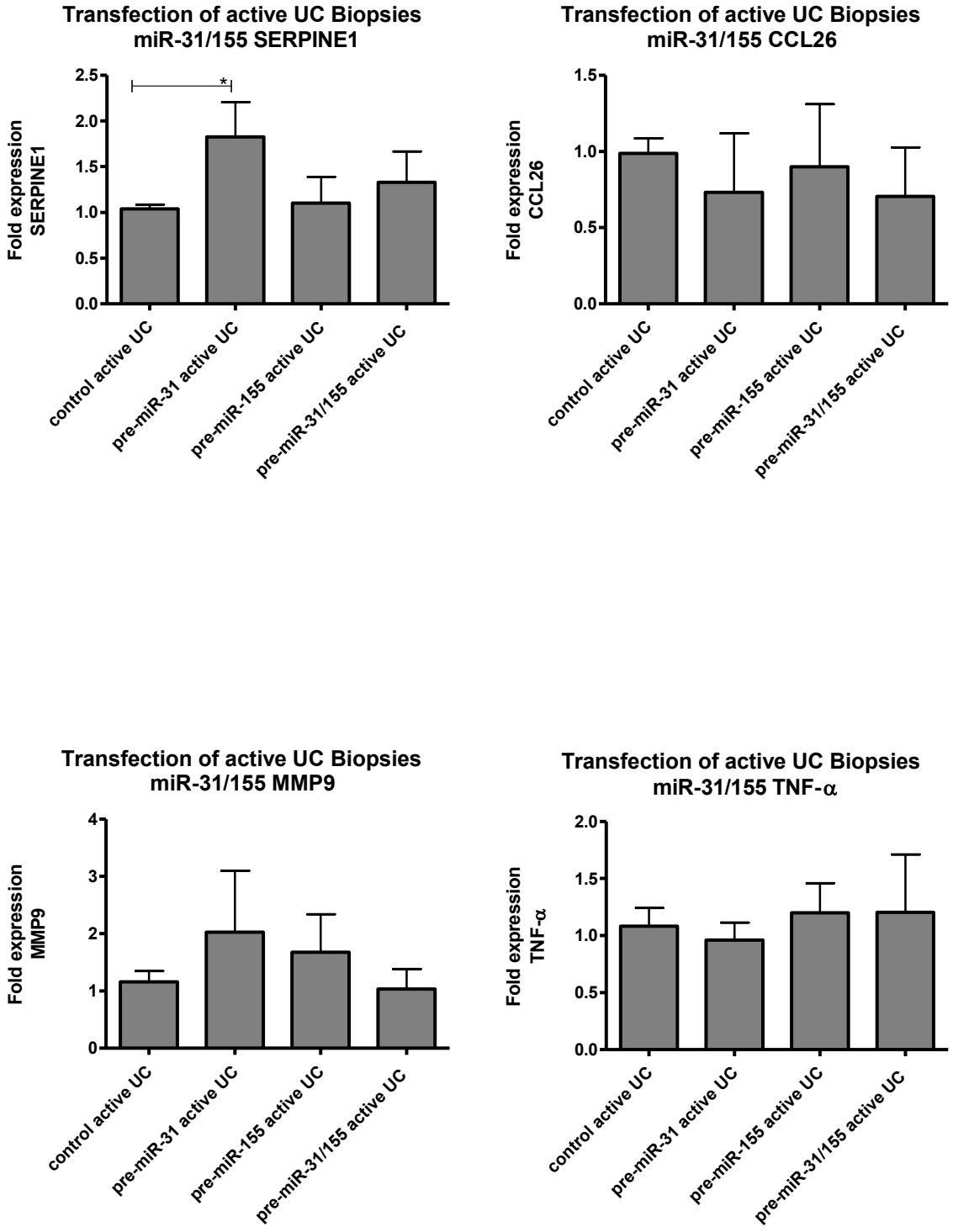


Figure 55. Effects of miR-31 and miR-155 transfection of active UC explant cultures on SERPINE1, CCL26, MMP9 and TNF-α. No clinically significant effect of transfection with miR-31 and miR-155 in active UC explant cultures was observed SERPINE1, CCL26, MMP9 and TNF-α. GAPDH was used as a normalizer gene. Fold expression was calculated to the average value of unstimulated controls. *: P-value<0.05, **: P-value<0.01, ***: P-value <0.001

6.3 Discussion

IL-13 dependent genes (CCL18, SOCS1) were found to be significantly up-regulated in active UC in our patient data along with a down-regulation of IL13Rα1, compared to unaffected inactive UC and normal colonic tissue samples from the sigmoid colon of our patients (***Figure 13***). Protein expression of IL13Rα1 was also significantly reduced in active UC as compared to normal controls and unaffected, inactive UC as demonstrated by Western blotting of biopsy samples (***Figures 14*** and ***15***). MiR-31 and miR-155 were also significantly raised in paired samples in the active paired UC group (endoscopic Mayo score 2-3) (***Figure 16***).

Similar results were observed for mRNA of epithelial cell isolates from normal controls, unaffected inactive and active UC showing a significant down-regulation of IL13Rα1 with corresponding high levels of miR-31 and miR-155 in the active samples. SOCS1 was up-regulated in the inflamed epithelial isolates in active UC (see ***Figures 17*** and ***18***).

We hypothesised, that the increase of miR-31 and miR-155 in actively inflamed UC was an attempt of auto-regulation to reduce inflammation by reducing the IL13Rα1, but may be ultimately unsuccessful due to the overwhelming inflammatory response in active UC.

Using these two micro-RNAs (miR-31 and miR-155 and their combination) to target IL13Rα1 to reduce its expression was successfully demonstrated in *in vitro* experiments in HT-29 colonic epithelial cells (***Figures 40, 41*** and ***42***), monocytic THP-1 cells (***Figures 47, 48*** and ***49***) and human monocytes/macrophage cell models (***Figures 50, 51*** and ***52***). The reduction of IL13Rα1 led to a significant reduction of expression of CCL18 (human monocytes), SOCS1 (HT-29 cells, THP-1 cells, human monocytes) and CCL26 (HT-29 cells) following stimulation with IL-13 in mRNA expression. We were also able to show a significant down-regulation of IL13Rα1 protein expression in epithelial colonic HT-29 cells by pre-miR-31 and pre-miR-155 and its combination which also led to a significant reduction in phosphorylation of STAT6 (***Figures 43*** and ***44***). Down-regulation of the JAK-STAT6 pathway with siRNAs has been shown to lead to a reduction of the toxic effects of IL-13 to epithelial cells previously. (Rosen, Frey et al. 2011).

Therefore the transfection of biopsy samples in explant cultures from patients with active UC was the logic conclusion to investigate, if these two microRNAs (miR-31 and miR-155) have therapeutic potential leading to reduction of pro-inflammatory effects of IL-13 pathway stimulation by reducing the IL13Rα1.

Transfection of active UC explant cultures (endoscopic Mayo score 2-3) with pre-miR-31 and pre-miR-155 and its combination clearly has an effect on IL13Rα1 significantly reducing its expression to about 60% compared to a control sample at a dose of 100nM in the single transfection samples with pre-miR-31 and pre-miR-155, and a further reduction of the mRNA expression to about 50% was observed in the combination treatment group of pre-miR-31/155 at 50nM each (***Figure 53***). This effect also translated into the expression of the IL-13 dependent gene CCL18 and SOCS1 reducing its mRNA expression in an even more pronounced magnitude. Especially the combination of pre-miR-31 and pre-miR-155 was able to reduce the expression of CCL18 mRNA to about 20% and the expression of SOCS1 to about 30% as compared to the control scramble pre-miR-control (***Figure 54***).

The reduction of IL13Rα1 by pre-miR-31 and pre-miR-155 and the combination of the two microRNAs confirms the findings in our in vitro cell models of the colonic epithelial cells (HT-29 cells), THP-1 cells and the human monocyte derived macrophages (***Figures 40, 49*** and ***52).***

The reduction of CCL18 corroborates with our findings in the in vitro experiments with human monocyte derived macrophages, which provided a model for macrophages and dendritic cells of the sub-mucosa in the gut (***Figure 50***).

The reduction of SOCS1 was seen in all of our in vitro cell lines and is in proportion with the reduction of IL13Rα1 mRNA (***Figures 41, 45*** and ***51***).

No significant down-regulations were seen in the mRNA levels of SERPINE1 and CCL26, which can be explained by the presence of TGF-β in the biopsy samples which also stimulates the expression of these genes apart from IL-13. We have shown the effects of TGF-β stimulation on SERPINE1 and CCL26 in HT-29 cells (***Figure 22***). It is therefore not surprising that the effects of the reduction of CCL26, by transfection with pre-miR-31 and pre-miR-155 and its combination, seen

in HT-29 cells could not be replicated as one would not expect any significant amount of TGF-β being present in an in vitro experiment.

MMP9 was seen to be increased in all microRNA treatment groups and may just generally represent higher levels of MMP9 due to tissue degradation releasing increased levels of MMP9 (***Figure 55***).and therefore no effect of the microRNAs on its expression was seen despite the significant effect on IL13Rα1 leading to its down-regulation.

No significant differences were seen in the expression of TNF-alpha as compared to the control group and may indicate that the IL13 pathway does not significantly influence the expression of TNF alpha (***Figure 55***).

The high levels of miR-31 and miR-155 in inflamed UC tissue causing a down-regulation of IL13Rα1 may well be an attempt to stop the activation of the IL-13/STAT6 pathway.
The potential anti-inflammatory effect of miR-31 and miR-155 through reduction of the IL13Rα1 expression leads to the conclusion that these microRNAs could have therapeutic use in ulcerative colitis.
As discussed before, high levels of SOCS1, an inhibitor of STAT6 activation, might reflect the first line of defence following increased IL-13 activation through IL13Rα1, especially considering its activation profile reaching the maximum expression at 1h (Hebenstreit, Luft et al. 2003). Reducing the expression of IL13Rα1 employing microRNAs targeting this receptor seems a more long-term strategy to prevent tissue damage.
As previously described, IL-13 signalling through IL13Rα1 activating STAT6 through its phosphorylation plays an important role in the pathogenesis of UC, which has been highlighted by studies showing that ultimate down-regulation of the JAK-STAT6 pathway with siRNAs or HDAC inhibitors leads to a reduction of the toxic effects of IL-13 to epithelial cells (Rosen, Frey et al. 2011). Interferon-β-1a, located in a chromosome cluster next to miR-31 gene (***Figure 4***), down-regulates STAT6 via activation SOCS1 to achieve clinical remission in a subset of patients with active UC (Mannon, Hornung et al. 2011).

Overall taking the in vitro data from HT-29 cells and human monocytes/macrophages into account showing the potential to down-regulate IL13Rα1, as well as the data from the Explant Culture biopsy transfection provides evidence of the therapeutic potential of miR-31 and miR-155 in UC.

7 Results 4: Ulcerative Colitis and Crohn's disease mRNA and microRNA expression profiling. Effect of therapy in inflammatory bowel disease on inflammatory genes and microRNA expression

7.1 MicroRNA and mRNA expression in UC

In order to analyse the immune profile of our patient samples depending on their inflammatory status, we pooled the samples under "unaffected, inactive" or "active" inflammation regardless of current medical treatment (see demographic data, ***Table 11***). Pinch biopsies were collected from unaffected, inactive and active mucosa in the sigmoid colon from patients with UC (see ***sections 2.7 and 2.8***). Unaffected, inactive samples in UC had an endoscopic Mayo score of 0-1 and active samples had an endoscopic Mayo score of greater or equal to 2 (see ***Table 4*** for Mayo scoring system). A total number of 56 sigmoid biopsies from UC patients (unaffected, inactive and active) were analysed. RNA was extracted using TRIzol (see ***2.16***) and assayed by RT-qPCR (see ***2.17 and 2.18***). Samples which showed lower than twice the standard deviation of the C_T value of all the samples in housekeeping genes (RNU44 for microRNA and GAPDH for mRNA) were excluded as indicative of RNA degradation or poor quantity/quality. Samples with similar C_T values for the housekeepers (RNU44 and GAPDH) were included in the analysis. The results are shown in ***Figure 56*** for microRNA and ***Figure 57*** (Th1 genes), ***Figure 59*** (Th2 genes) and ***Figure 60*** (tissue remodelling genes) for mRNA.

7.1.1 Introduction

CD is a mainly Th1 and Th17 mediated process, whereas UC seems to be predominately mediated through Th2 and NK T-cells producing IL-13 (Shih and Targan 2008, Matricon, Barnich et al. 2010, MacDonald, Monteleone et al. 2011, Strober and Fuss 2011). Severe mucosal tissue damage requiring efficient wound healing is a main feature of inflammatory bowel disease (IBD), important in both Crohn's disease and ulcerative colitis. The imbalance between healing and inflammation is characterised by formation of ulcers and fistulas the one hand, and of fibrosis and strictures on the other, underlining the complex nature of tissue remodelling in IBD (Rieder, Brenmoehl et al. 2007).

Little is known about the effects of IBD treatments on the expression of pro-inflammatory, immuno-regulating and tissue remodelling pathways commonly deregulated in IBD, both in Crohn's disease and Ulcerative Colitis. No data is available regarding the possible differential expression of microRNAs in IBD tissues influenced by the various treatments available in IBD. Previous studies of microRNAs in IBD have not focused on IBD treatments, which could potentially alter the expression of microRNAs given their involvement in many biological processes.

Current drug treatments in IBD aim to induce and maintain the patient in remission and ameliorate the disease's secondary effects. Generally medications used in the treatment of IBD are 5-ASA (5-Amino Salicylates) preparations such as mesalazine (mainly in UC) (Camma, Giunta et al. 1997), topical and systemic steroids (Ford, Bernstein et al. 2011), the frequently used immune modulators such as the Thiopurines 6-mercaptopurin and azathioprine (Fraser, Orchard et al. 2002). Anti-TNF-α agents such as Infliximab and Adilumimab have given new options of treatment in the last few years (D'Haens, Panaccione et al. 2011).

Multiple mechanism of action have been demonstrated for 5-ASA compounds in vitro such as inhibition of the production of pro-inflammatory cytokines such as IL-1, TNF-α and inhibition of the lipoxygenase pathway, scavenging of free radicals and oxidants contributing to cellular detoxification as well as inhibition of the inflammatory-related Nuclear Factor κB (NF-κB) pathway (Barnes and Karin 1997) and inhibition of T-cell proliferation (Fujiwara, Mitsui et al. 1990).

A role for mesalazine as an additional ligand of PPAR-γ has been suggested, which may explain some of its therapeutic effects in UC (Rousseaux, Lefebvre et al. 2005). The direct effect in vivo on cytokines and microRNA expression in colonic mucosa in patients with unaffected, inactive and active ulcerative colitis is unknown.

6-Mercaptopurine and its pro-drug azathioprine (AZA) are purine analogues that are converted into 6-thioguanine nucleotides, which are the therapeutically active metabolites. 6-thioguanine nucleotides interfere with nucleic acid synthesis, exhibit anti-proliferative effects on activated lymphocytes and have been shown to induce T-cell apoptosis (Tiede, Fritz et al. 2003, Schroll, Sarlette et al. 2005). Their effect on the cytokine and microRNA profile of inflammatory bowel disease has not been determined to date.

Infliximab (IFX) is a chimeric (75% mouse/25% human) anti-TNF-α monoclonal antibody and Adilumimab is a fully humanised anti-TNF-α monoclonal antibody. Ljung et al reported a decreased immune-histochemical expression of TNF-α, IL-1β and INF-γ in mucosal biopsies in patients with Crohn's disease after treatment with infliximab (Ljung, Axelsson et al. 2007). In UC patients, IFX induced down-regulation of the mucosal TNF-α and IFN-γ mRNA expression. Moreover, T lymphocyte and macrophage numbers were significantly decreased in patients with endoscopically healed mucosa after Infliximab treatment (Olsen, Cui et al. 2009).

Extensive immune profiling of UC and CD has been undertaken by Christophi et al. Their group analysed 70 selected immune genes, important in IBD signalling, from formalin-fixed, paraffin-embedded colon biopsy samples from normal control subjects, UC and CD patients having either severe colitis or quiescent disease (n = 98 subjects). Expression levels of signalling molecules including IL-6/10/12/13/17/23/33, STAT1/3/6, T-bet, GATA3, Foxp3, SOCS1/3, and downstream inflammatory mediators such as chemokines CCL-2/11/17/20, oxidative stress inducers, proteases, and mucosal genes were shown to be differentially regulated between UC and CD and between active and quiescent disease. A possible role of novel genes in IBD, including SHP-1, IRF-1, TARC, Eotaxin, NOX2, arginase I, and ADAM 8 was also documented (Christophi, Rong et al. 2012). Although extensive, this study did not take different medications patients were on into account and was performed on paraffin fixed specimens as opposed to fresh samples.

The impact of IBD treatments on cytokine signalling pathways in colonic biopsies from IBD patients is not well documented; therefore the influence of various medications on these pathways is largely unknown. Investigating the effect of IBD therapy on pathways deregulated in

IBD could give a new insight in treatment choice and may be able to predict treatment failure. Moreover, relating changes in mRNA and microRNAs levels may also give insight into treatment outcome and patient responsiveness to medication.

MiR-31, miR-155 and miR-146a were chosen in this project due to their extensive involvement in pro-inflammatory pathways deemed to be contributing to the pathogenesis of IBD and their contribution to the regulation of T-cell biology. As outlined above treatments commonly used in IBD such as 5-ASA, Thiopurines and anti-TNF-α compounds all interfere with pro-inflammatory pathways and T-cell biology.

MiR-31 and miR-155 have both been shown to be deregulated in UC. MiR-31 has also been demonstrated to be elevated in CD (Fasseu, Treton et al. 2010, Takagi, Naito et al. 2010, Olaru, Selaru et al. 2011).

MiR-31 has a wide variety of predicted gene targets such as FOXP3, CD28, HDAC1, HDAC2 and HDAC9 (Rouas, Fayyad-Kazan et al. 2009). MiR-31 has been suggested to play an important role in inflammation preventing leucocyte recruitment (Suarez, Wang et al. 2010) and T-reg regulation (Rouas, Fayyad-Kazan et al. 2009) as well as exhibiting multiple functions in neoplastic disease (Schmittgen 2010, Stuelten and Salomon 2010, Olaru, Selaru et al. 2011).

MiR-155, together with miR-146 and miR-132, is significantly involved in innate immunity by regulating the acute inflammatory response after pathogen recognition by Toll-like receptors (TLRs) on monocytes or macrophages (Bakirtzi, Hatziapostolou et al. 2011). Expression of miR-155 was observed to be inducible during bacterial stimulation, as well as after exposure of cells to pro-inflammatory cytokines such as IFN-γ, IFN-β or TNF-α (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007). A positive correlation between miR-155 up-regulation and NF-κB activation has been reported providing a possible involvement between inflammation and cancer in IBD patients (Ma, Becker Buscaglia et al. 2011). MiR-155 maintains T-regs cell proliferation and homeostasis by the down-regulation of SOCS1 expression and also contributes to their development by directly targeting Foxp3 (Rodriguez, Vigorito et al. 2007, Kohlhaas, Garden et al. 2009). MiR-155 has also been shown to be involved in B-cell development and function (Chen, Li et al. 2004, Hu, Fong et al. 2010). MiR-155 directly targets and decreases levels of suppressor of cytokine signalling 1 (SOCS1) and negatively regulates the Toll-like receptor pathway affecting the STAT6 pathway (Wang, Hou et al. 2010). Data presented in this

project showed that both, miR-31 and miR-155, as well as their combination, are able to down-regulate IL-13 dependant genes through down-regulation of IL13Rα1 (see ***Figure 56***).

MiR-146a and miR-155 can be over expressed by inflammatory mediators including NF-κβ and microbial components and influence the course of inflammation (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007). MiR-146a deficiency in T-regs has also been shown to result in inability to exhibit their suppressor function and IFN-γ response deregulation through an increase in Signal Transducer and Activator 1 (STAT1) expression and activation (Lu, Boldin et al. 2010). miR-146a has been demonstrated to inhibit the expression of IRAK1 and TRAF6, impair NF-κB activity and suppress the expression of NF-κB target genes such as IL-6, IL-8, IL-1β and TNF-α (Taganov, Boldin et al. 2006, (Bhaumik, Scott et al. 2008).

MiR-146a has not been yet reported to be increased in inflammatory bowel disease.

We assessed the levels of microRNAs 31, 155 and 146a and inflammatory mediators in sigmoid biopsies of UC and CD patients independently of the medication they were subjected to, but divided the groups into “unaffected, inactive” and “active” disease. We then subdivided the patients into unaffected, inactive and active medication groups to assess the possible effect of individual drugs on microRNA and gene expression and compared the samples to UC and CD patients who were treatment naïve. mRNA expression of pro-inflammatory genes and tissue remodelling genes were divided into a Th1 (TNF-α, IL12βR1, IL12βR2, IL1β, IL2β and INF-γ), Th2 (CCL18, SOCS1, CCL26, IL13Rα1 and TSLP) and a tissue remodelling panel (SERPINE1, MMP9, MMP12, COL1A2 and TGF-β1) and were analysed firstly in the total group of UC and CD, and then subdivided according to medication exposure for UC and CD.

TNF-α mRNA gene expression in inflamed colorectal mucosa in UC has been shown by qPCR and was confirmed by immunohistochemistry (Harvey, Naciti et al. 2004). Raised TNF-α in inflamed mucosa of CD patients has been reported and has since been a cornerstone in the pathogenesis of CD (Reimund, Wittersheim et al. 1996) and triggered the development of anti-TNF therapy.

The ACCENT I and II trials showed superior remission rates in mucosal healing and closure of fistulae in patients with Crohn’s disease treated with Infliximab compared to placebo (Hanauer, Feagan et al. 2002, Sands, Anderson et al. 2004, Sands, Blank et al. 2004).

ACT 1 and ACT 2 studies (Infliximab), as well as the CHARM trial (Adalimumab) demonstrated a beneficial effect of anti-TNF therapy in UC (Rutgeerts, Sandborn et al. 2005, Colombel, Sandborn et al. 2007) confirming the importance of TNF-α in the pathogenesis of UC. Infliximab was shown to alter the expression profile of mRNA of genes involved in the inflammatory response, cell-mediated immune responses and mucosal barrier function in patients with UC who responded to treatment (Arijs, Li et al. 2009, Toedter, Li et al. 2011, Toedter, Li et al. 2012).

The T cell response to IL-12 is dependent on the expression of high affinity IL-12R composed of two subunits, termed IL-12Rβ1 and IL-12Rβ2. Th1 cells express both the IL-12Rβ1 and IL-12Rβ2 subunits, whereas Th2 cells express only the IL-12Rβ1 chain (Presky, Yang et al. 1996). IL-12Rβ2 has been shown to be up-regulated in CD (Parrello, Monteleone et al. 2000).

IL-1β and IL-2 were shown to be increased in colonic tissue in both ulcerative colitis and Crohn's disease patients (Brynskov, Tvede et al. 1992). IL1β was also demonstrated to be increased in macroscopically normal tissue in CD (Reimund, Wittersheim et al. 1996).

IFN-γ secreting lamina propria lymphocytes are abundant in the mucosa of patients with Crohn's disease (Kugathasan, Saubermann et al. 2007), but there is a distinctive lack of IFN-γ expression in UC (Strober and Fuss 2011).

CCL18 is known to be up-regulated in various tissues in an inflammatory environment, such as skin, lungs and lymphatic tissue. It might induce expression of chemokines to attract lymphocytes, immature DC and monocytes toward various sites where macrophages and DC participate in Th1- or Th2-mediated immune responses (Strulovici-Barel, Omberg et al. 2010). CCL18 expression is regulated by the Th2 cytokines IL-4 and IL-13 and IL-10 (Meau-Petit, Tasseau et al. 2010). CCL18 expression in IBD has not been reported yet.

SOCS1 is an inhibitor of STAT6 via stimulation of IL13Rα1 through IL-4 and IL-13 (Dickensheets, Venkataraman et al. 1999). Type 1 interferons have been shown to influence STAT6 expression via SOCS1 by down-regulation of IL-13 and IL-4 in macrophages (Dickensheets, Venkataraman et al. 1999). In a clinical setting Interferon-β-1a has been shown to induce a clinical response and remission in a large subset of patients with UC by inhibition of IL-13 production facilitated by SOCS proteins (Mannon, Hornung et al. 2011). This highlights the potential role of SOCS1 in UC. SOCS-1 mRNA is induced by and inhibits also a variety of Th1 cytokines, including IFN-γ and TNF-α (Greenhalgh, Miller et al. 2002).

Eotaxin-3 (CCL26) is a Th2 driven cytokine whose expression is stimulated by IL-13 through the IL13Rα1. CCL11/eotaxin-1, CCL24/eotaxin-2 and CCL26/eotaxin-3 are known to attract CCR3-expressing Th2-polarized lymphocytes. A recent study revealed raised CCL26 levels in blood and colonic biopsies of patients with UC as compared to normal controls (Manousou, Kolios et al. 2010).

Increased expression of IL13Rα1 and IL-13Rα2 were previously reported in patients with UC who underwent colectomy (Mandal and Levine 2010), but no grade of inflammation, no reason for colectomy was or any other clinical, such as whether patients received rescue therapy for severe colitis or underwent colectomy for dysplasia or cancer was given. IL-13 has been implicated to play an important role in fistula formation in Crohn's disease (Scharl, Frei et al. 2013).
TSLP is an inflammatory Th2 regulator that is increased in the mucosal lesions of UC and increased TSLP expression by IECs may trigger exacerbation of UC (Tanaka, Saga et al. 2010).

SERPINE1 has been shown to be a crucial player in wound healing and high levels of SERPINE1 blocks smooth muscle migration and therefore impairs wound healing (Holubar and Harvey-Banchik 2007). SERPINE1 has been shown to be up-regulated by IL-13 (Martinez-Nunez, Louafi et al. 2011). Increased SERPINE1 levels have been described in UC and CD resection specimens (Miseljic, Galandiuk et al. 1995).

MMP9 is up-regulated in IBD (Garg, Vijay-Kumar et al. 2009) and MMP12 was shown to be abundantly expressed by plump-macrophage-like cells of the inflamed lamina propria in ulcerative colitis (Vaalamo, Karjalainen-Lindsberg et al. 1998). MMP9 and MMP12 have been both implicated in Th2 driven IL-13 dependent inflammation and fibrosis in mouse models of liver and lung (Madala, Pesce et al. 2010).

TGF-β1 was shown to be elevated in both active CD and active UC compared to normal controls and unaffected, inactive CD and UC samples. No clinical data was given as to which medication patients were taking (Babyatsky, Rossiter et al. 1996).

COL1A2 has been shown to be up-regulated in a mouse model of colitis and was not down-regulated by blockade of NF-kB (Wu and Chakravarti 2007).

This is a comprehensive study of the effect of current treatments in IBD on the expression profile of microRNAs (miR-31, miR-155 and miR-146a) involved in pro-inflammatory and immunological processes and Th1, Th2 and tissue remodelling genes in UC and CD. After establishing an overall profile of these microRNAs and Th1, Th2 and tissue remodelling genes in the total number of unaffected, inactive and active UC and CD sigmoid biopsies, an analysis of the impact of drug treatments on these parameters will be established, comparing treatments to treatment naïve samples.

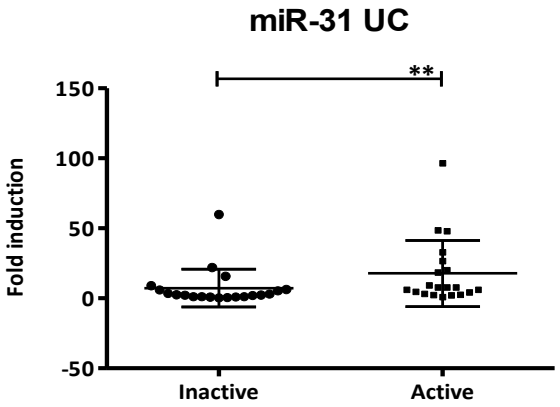
7.1.2 MicroRNAs 155, 31 and 146a are dysregulated in UC

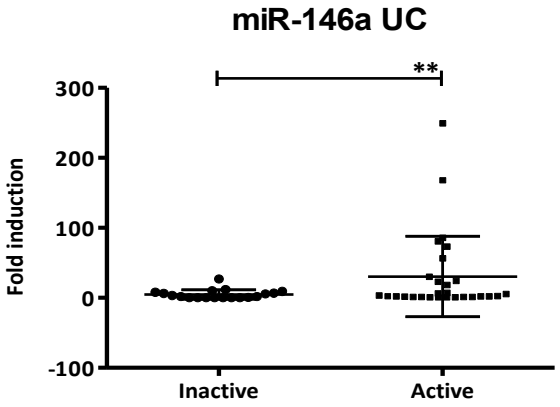
Table 15 shows the sample size of each group of the total study population after exclusion of poor quality samples with lower than twice the standard deviation of the Ct value of all the samples for RNU44.

MicroRNA	**Unaffected, inactive**	**Active**
miR-31	20	20
miR-155	32	24
miR-146a	19	28

Table 15. Sample sizes for microRNA analysis in samples from patients with unaffected, inactive and active UC.

MiR-31, miR-155 and miR-146a expression was significantly up regulated in active samples compared to unaffected, inactive samples of UC patient ***(Figure 56).***





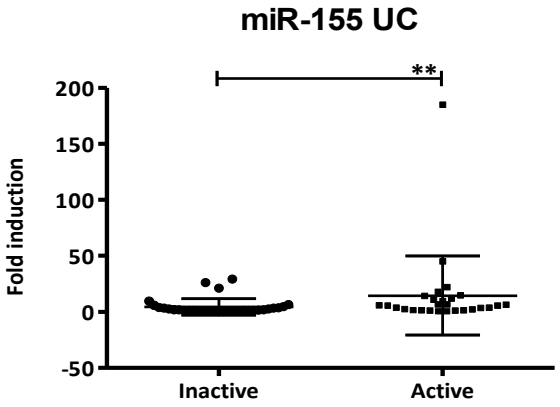


Figure 56. MicroRNAs 31, 155 and 146a expression in unaffected, inactive vs active UC samples. microRNA expression was determined by RT-qPCR and fold induction calculated using RNU44 as housekeeper. Mann-Whitney t-test was performed.*= P-value<0.05, **= P-value<0.01, ***= P-value<0.001, ****= P-value<0.0001

7.1.3 mRNA expression profile of UC patients

After determining that microRNAs involved in the immune response and inflammation were significantly up-regulated in actively inflamed sigmoid biopsies of UC patients, we aimed to establish an immune/tissue remodelling gene expression profile in those samples. Sample sizes were N=15-20 for each group. We grouped the analysed genes into Th1-related, Th2-related and fibrosis/remodelling-related genes in ***Figures 57, 58*** and ***59***, respectively.

Our panel of Th1-related genes included TNF-□, IL12βR1, IL1β and IL2β. Only IL1β showed a significant up regulation in samples from patients with active UC (***Figure 57***).

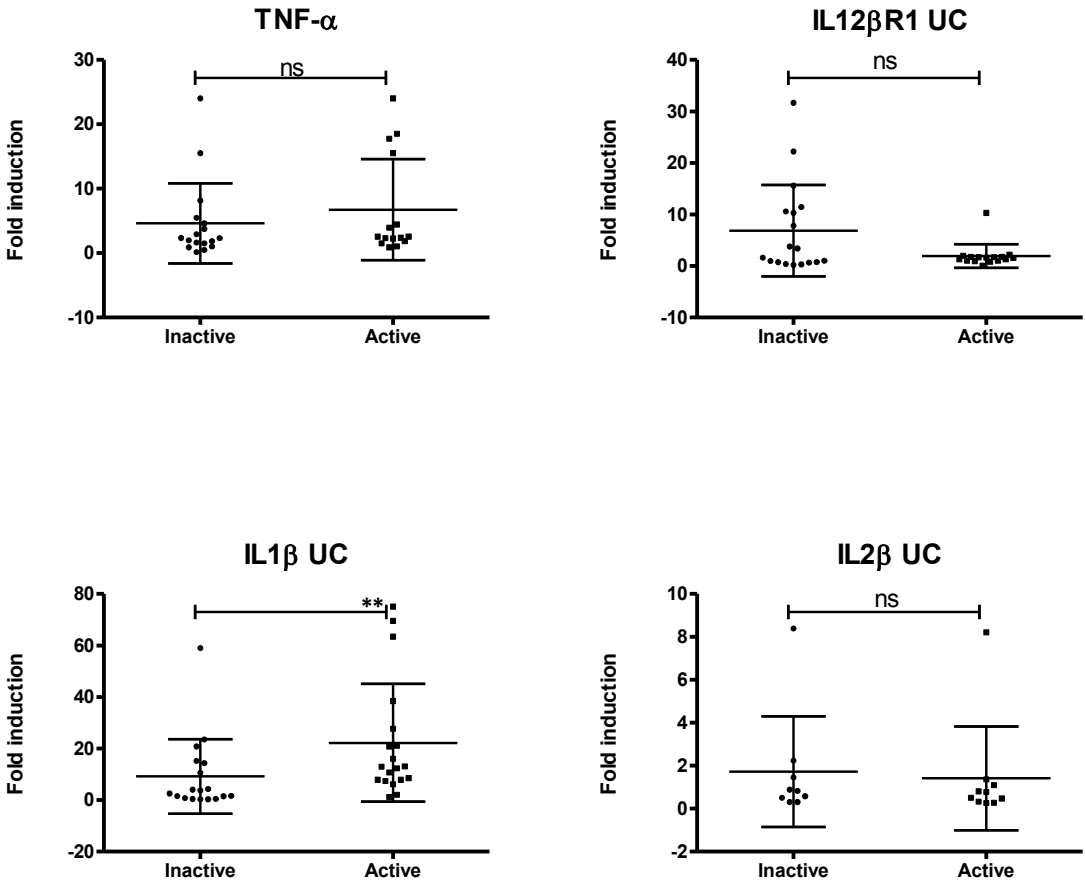


Figure 57. Th1-related genes analysed in active and unaffected, inactive UC samples. mRNA expression was determined by RT-qPCR and fold induction calculated using GAPDH as housekeeper. Mann-Whitney t-test was performed.*= P-value<0.05, **= P-value<0.01, ***= P-value<0.001, ****= P-value<0.0001

We then analysed a panel of Th2-linked genes: CCL18, SOCS1, CCL26, IL13Rα1 and TSLP. CCL18, SOCS1 and CCL26 were found significantly increased in active disease, while IL13Rα1 was significantly down-regulated. TSLP was not significantly different (***Figure 58***).

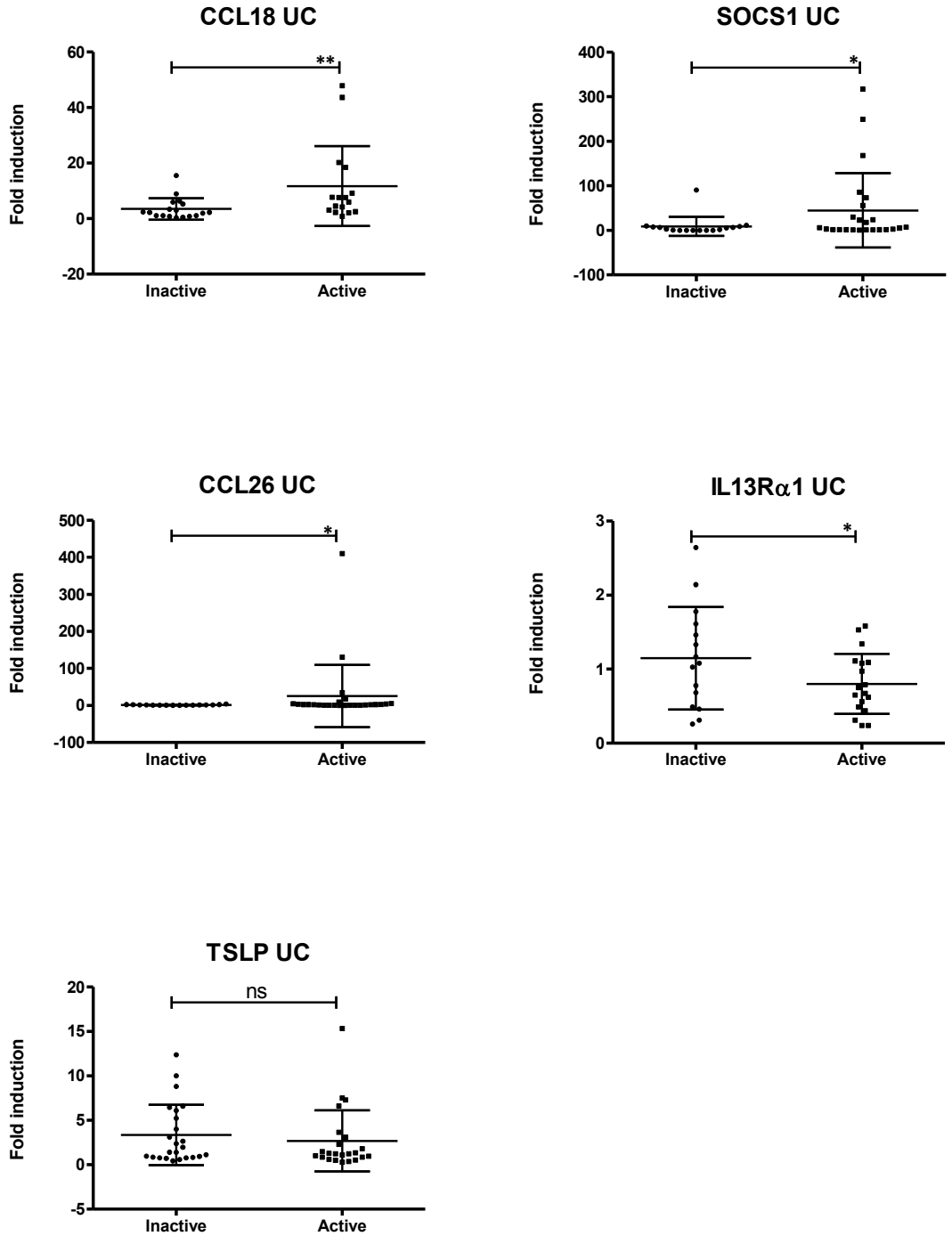


Figure 58. Th2-related genes analyzed in active and unaffected, inactive UC samples. mRNA expression was determined by RT-qPCR and fold induction calculated using GAPDH as housekeeper. Mann-Whitney t-test was performed.*= P-value<0.05, **= P-value<0.01, ***= P-value<0.001, ****= P-value<0.0001

Genes involved in tissue remodelling showed significant up-regulation in active UC sigmoid biopsies were SERPINE1, MMP9 and MMP12. COL1A2 and TGF-β1 were not significantly different in unaffected, inactive and active UC biopsies (***Figure 59***).

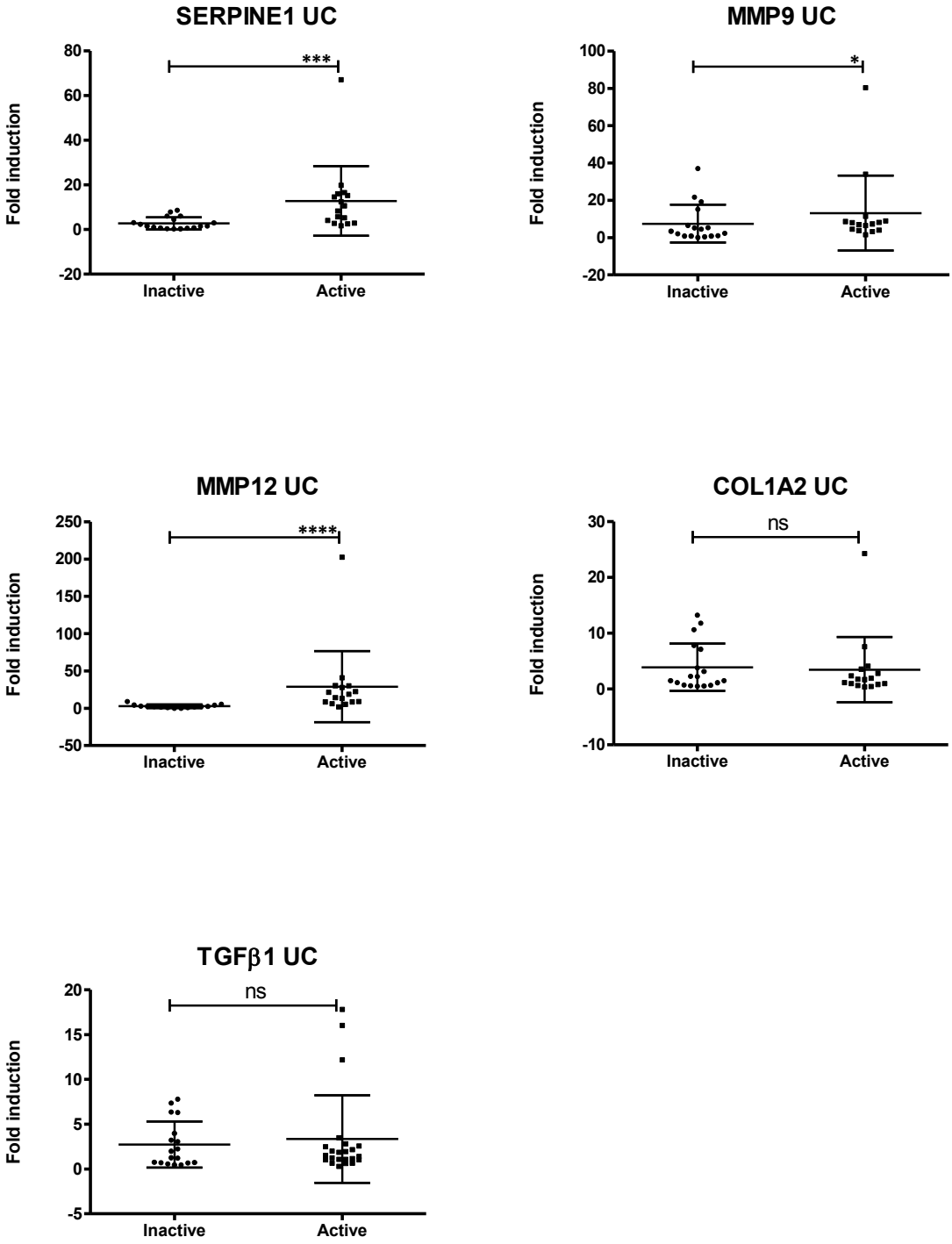


Figure 59. Fibrosis/remodelling-related genes analysed in active and unaffected, inactive UC samples. mRNA expression was determined by RT-qPCR and fold induction calculated using GAPDH as housekeeper. Mann-Whitney t-test was performed.*= P-value<0.05, **= P-value<0.01, ***= P-value<0.001, ****= P-value<0.0001

7.1.4 5-ASA and Thiopurine modulation of microRNA and mRNA expression in UC

7.1.4.1 Introduction

To investigate the effects of medication on the microRNA and inflammatory/tissue remodelling mRNA expression profile of UC patients, we subdivided the samples from unaffected, inactive and active UC tissue and grouped them according to their drug treatment, 5-ASA or Thiopurines (TP), respectively. Each group contained a similar number of samples (sample sizes are indicated in ***Table 17***) and fold induction was calculated comparing to treatment naïve unaffected, inactive UC. RNA was extracted using TRIzol (see ***section 2.16***) and analyzed by RT-qPCR (see ***2.17*** and ***2.18***). MicroRNAs miR-31, miR-146a and miR-155 expression was assayed (***Figure 60***). Messenger RNA expression profiles are shown in ***Figures 61 to 66***. Within each medication group, genes were sub-grouped according to their general function: Th1 (***Figures 61*** and ***62*** for 5-ASA and TP, respectively), Th2 (***Figures 63*** and ***64*** for 5-ASA and TP, respectively) and fibrosis/tissue remodelling (***Figures 65*** and ***66*** for 5-ASA and TP, respectively).

7.1.4.2 microRNAs 31, 155 and 146a are modulated by 5-ASA and Thiopurines in UC

We analysed the expression of miR-31, miR-146a and miR-155 by RT-qPCR (see ***2.16, 2.17*** and ***2.18***) in unaffected, inactive or active UC sigmoid biopsies from treatment naïve (***Nil***) and likewise undergoing either 5-ASA or Thiopurine treatment. Sample size is indicated in ***Table 16***. ***Figure 60*** shows the results.

	Nil		**5-ASA**		**TP**	
MicroRNA	Unaffected, inactive	Active	Unaffected, inactive	Active	Unaffected, inactive	Active
miR-31	7	7	5	7	7	6
miR-146a	4	7	7	7	7	6
miR-155	10	10	10	7	8	11

Table 16. Sample sizes for microRNA expression analysis in UC patients untreated and undergoing 5-ASA or Thiopurine treatment.

We aimed to determine whether microRNA levels changed with drug treatment in different states of the disease. A possible connection between microRNA expression and medication effectiveness may give insight on response rates to different drugs and could potentially be used as biomarkers for the treatment of UC.

MiR-31 expression levels were found to be significantly up-regulated in active compared to unaffected, inactive UC samples of treatment naïve patients (Nil) (***Figure 60***) in keeping with previous results in the total expression of miR-31 in the pooled samples ***(Figure 56***). Both 5-ASA and Thiopurines significantly increase miR-31 expression when compared to unaffected, inactive treatment naïve patients (***Figure 56***). Similar to the findings in miR-155 the significant difference between unaffected, inactive and active 5-ASA and Thiopurine treated patients was lost as a result of treatment.

Interestingly, patients not responding to 5-ASA or Thiopurines (***Figure 60***) lost the significant up-regulation of miR-31 and miR-155 compared to treatment naïve patients with active disease and may therefore fail to down-regulate the IL13Rα1.

MiR-146a levels were shown to be significantly up-regulated in active treatment naïve samples compared to unaffected, inactive untreated samples (***Figure 60***), in keeping with our previous results in pooled samples (***Figure 56***). 5-ASA and Thiopurines increased miR-146a levels in both unaffected, inactive and active UC (***Figure 60***). Similar to the results of miR-31 and miR-155, the significant difference of miR-146a expression between unaffected, inactive and active 5-ASA and Thiopurine treated samples was lost.

Figure 60 shows that the significant difference in miR-155 expression between treatment naïve unaffected, inactive and active UC is abolished by 5-ASA and Thiopurines. Levels of miR-155 are significantly increased in 5-ASA treatment groups when compared to treatment naïve unaffected, inactive samples. MiR-155 levels in active disease treated with 5-ASA or Thiopurines were lower than in treatment naïve patients with active disease.

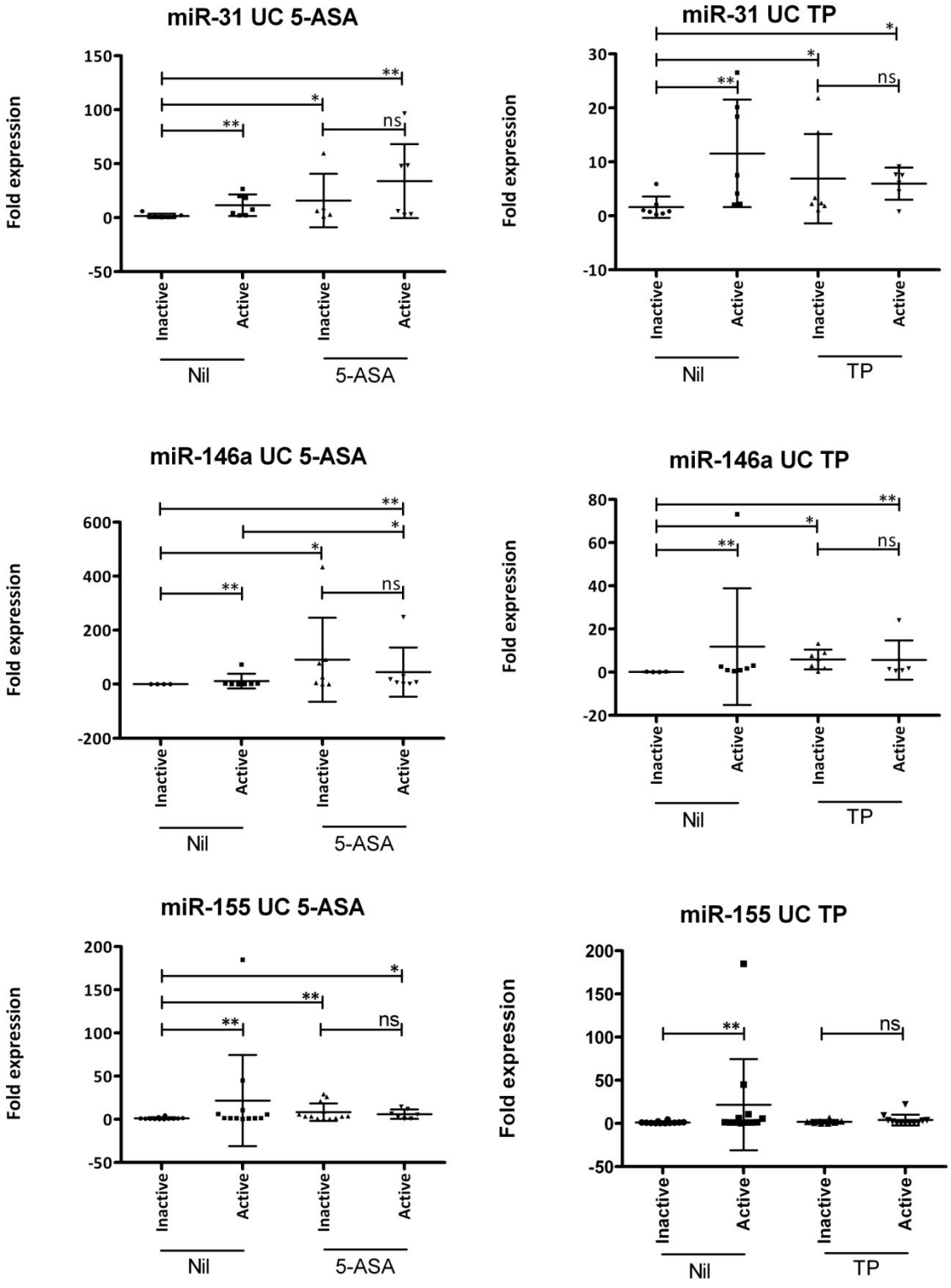


Figure 60. MiR-31, miR-146a and miR-155 expression in unaffected, inactive and active UC samples from treatment naïve patients (Nil) or undergoing 5-aminosalicylic acid (5-ASA) or Thiopurine (TP). MicroRNA expression was determined by RT-qPCR. Mann-Whitney t-test was performed. *:P-value<0.05, ** P-value<0.01, *** P-value<0.001.

7.1.4.3 UC mRNA expression is modulated by 5-ASA and Thiopurine treatment

After analysing the effects of 5-ASA and Thiopurines on the expression profile of miR-31, miR-155 and miR-146a, mRNA expression levels of genes involved in inflammatory pathways and tissue remodelling in UC were assayed. Sample sizes are indicated in ***Table 17***. Samples were divided according to medication (5-ASA or TP). Th1, Th2 and fibrosis/remodelling gene expression was analysed. Results are shown in ***Figure 61*** (Th1 5-ASA), ***Figure 62*** (Th1 TP), ***Figure 63*** (Th2 5-ASA), ***Figure 64*** (Th2 TP), ***Figure 65*** (fibrosis/remodelling 5-ASA) and ***Fig.66*** (fibrosis/remodelling TP).

Nil		**5-ASA**		**TP**	
Unaffected, inactive	Active	Unaffected, inactive	Active	Unaffected, inactive	Active
6	6	5	5	6	6

Table 17. Sample sizes for mRNA expression analysis in UC patients untreated and undergoing 5-ASA or Thiopurines.

The Th1 panel of genes included TNF-, IL1β, and IL12Rβ1. IL1β, a pro-inflammatory/Th1 cytokine, was significantly up-regulated in treatment naïve active UC patients as compared to unaffected, inactive UC. The significant difference observed in unaffected, inactive and active treatment naïve samples was lost in both treatment groups, showing lower levels of IL1β in active disease as compared to treatment naïve active samples. 5-ASA treatment significantly down-regulated IL1β in treatment responders (***Figure 61***), while Thiopurines significantly increased IL1β expression in active and unaffected, inactive UC compared to unaffected, inactive treatment naïve patients, but still at lower levels as compared to active treatment naïve patients (***Figure 62***).TNF-α expression was not found significantly different between unaffected, inactive and active treatment naïve patients or treatment groups with 5-ASA or Thiopurines. In the treatment groups with 5-ASA and Thiopurines the expression of TNF-α in both unaffected, inactive and active disease is significantly increased compared to treatment naïve unaffected, inactive samples (***Figure 61*** and ***Figure 62***).IL12Rβ1 was not found significantly different or affected by either drug (***Figures 61*** and ***62***).

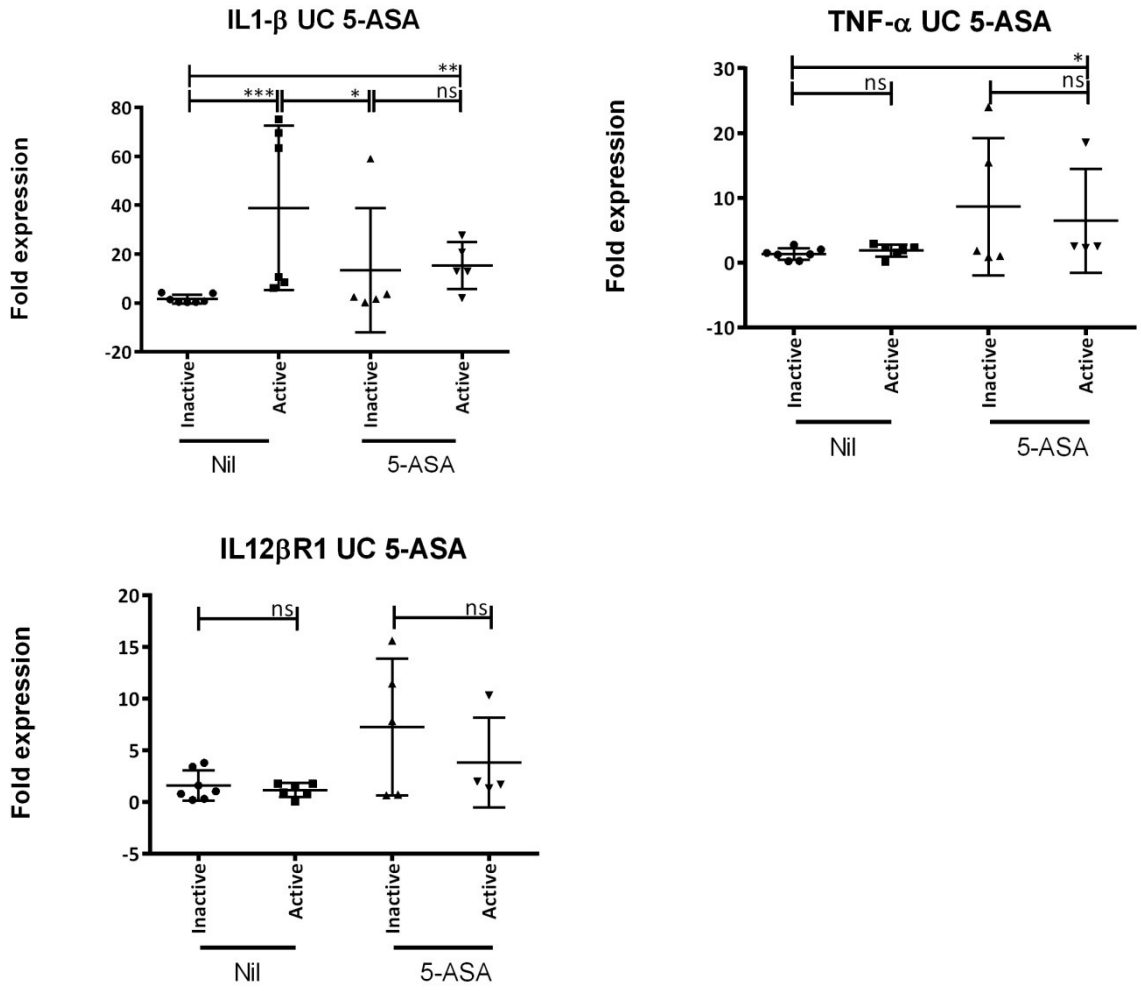


***Figure 61**. mRNA expression profile of Th1 related genes in active and unaffected, inactive UC samples from patients untreated (Nil) or undergoing 5-aminosalicylic acid (5-ASA) treatment. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *: P-value<0.05, ** P-value<0.01, *** P-value<0.001.*

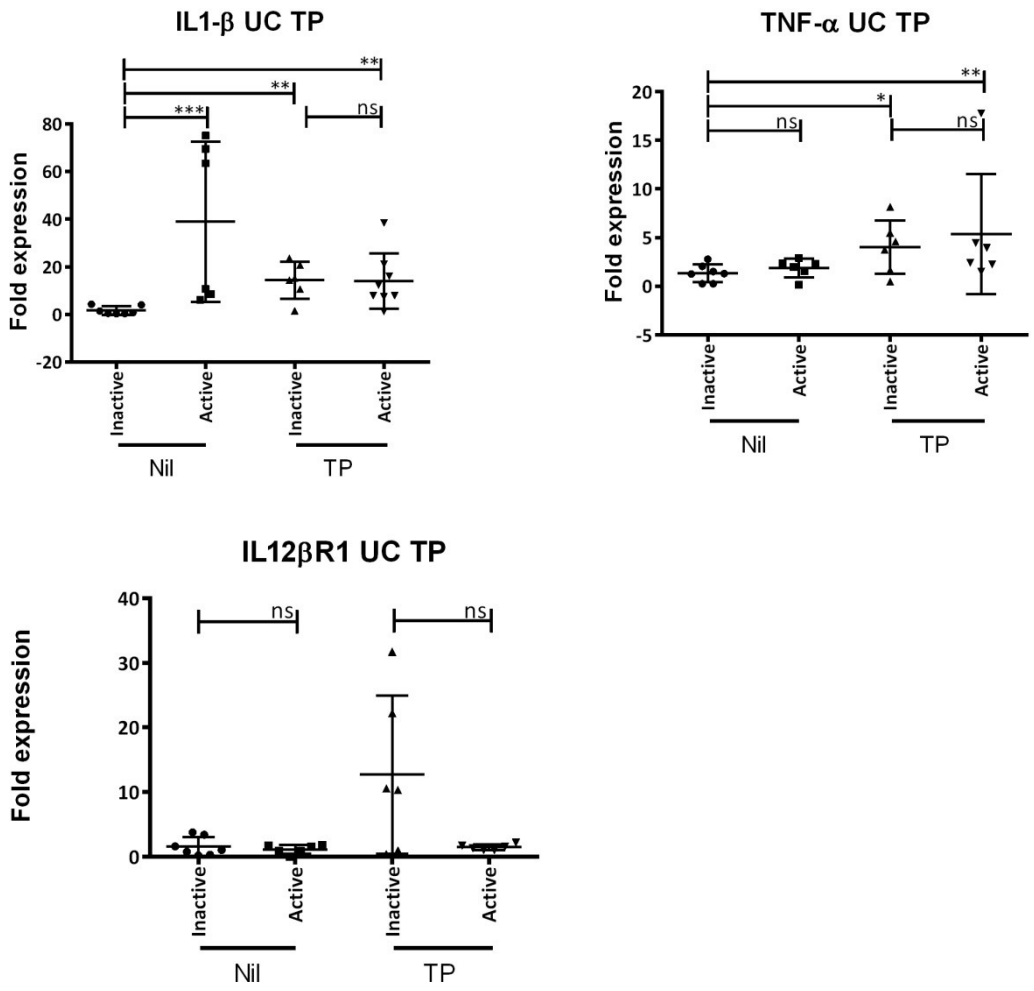


Figure 62. mRNA expression profile of Th1- related genes in active and unaffected, inactive UC samples from patients untreated (Nil) or undergoing Thiopurine (TP) treatment. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *: P-value<0.05, ** P-value<0.01, *** P-value<0.001.

The panel of Th2-related genes included CCL18, SOCS1, CCL26, IL13Rα1 and TSLP. CCL18, an IL-4/IL-13-dependent chemokine, was found significantly up regulated in active compared to unaffected, inactive treatment naïve samples (***Figures 63***and ***64***, "***Nil***"). 5-ASA and AZA seemed to have a similar effect on CCL18 expression increasing the CCL18 levels significantly in both unaffected, inactive and active treatment groups compared to treatment naïve unaffected, inactive UC samples. Treatment with both 5-ASA and Thiopurine abolished the significant difference between unaffected, inactive and active UC CCL18 levels observed in the treatment naïve group (***Figures 63*** and ***64***, respectively).

SOCS1 was significantly increased in active treatment naïve samples compared to unaffected, inactive untreated samples. SOCS1 was also significantly up-regulated in both unaffected, inactive and active treatment groups with 5-ASA and Thiopurines, when compared to treatment naïve unaffected, inactive samples. The significant difference between treatment naïve unaffected, inactive and active samples was lost in the treatment groups (***Figures 63*** and ***64***). The increase of SOCS1, an inhibitory of cytokine signalling, in both treatment groups is an interesting observation which might explain part of the therapeutic action of these drugs in UC. MiR-155, which directly targets SOCS1 (Wang, Hou et al. 2010), showed no increase in active treatment groups with 5-ASA and Thiopurines, which might be a sign of treatment failure.

No significant changes in treatment naïve or treatment groups were observed in CCL26 assays.

IL13Rα1 results showed down-regulation in active samples in treatment naïve samples when compared to unaffected, inactive samples. 5-ASA treatment showed no difference in treatment responders compared to unaffected, inactive treatment naïve samples (***Figure 63***). However, significant down-regulation observed in inflamed untreated samples was maintained in treatment responders in the Thiopurine group, but lost in non-responders to Thiopurines (***Figure 64***) with corresponding lower levels of miR-31 and miR-155 as compared to patients who respond to Azathioprine or 6-Mercaptopurine (***Figure 60***).

TSLP expression was not significantly different in treatment naïve UC patients in unaffected, inactive and active disease. Its expression was not influenced by 5-ASA treatment (***Figure 63***). However, treatment with Thiopurines increased the level of TSLP significantly in responders and was significantly reduced in non-responders to Thiopurines (***Figure 64***).

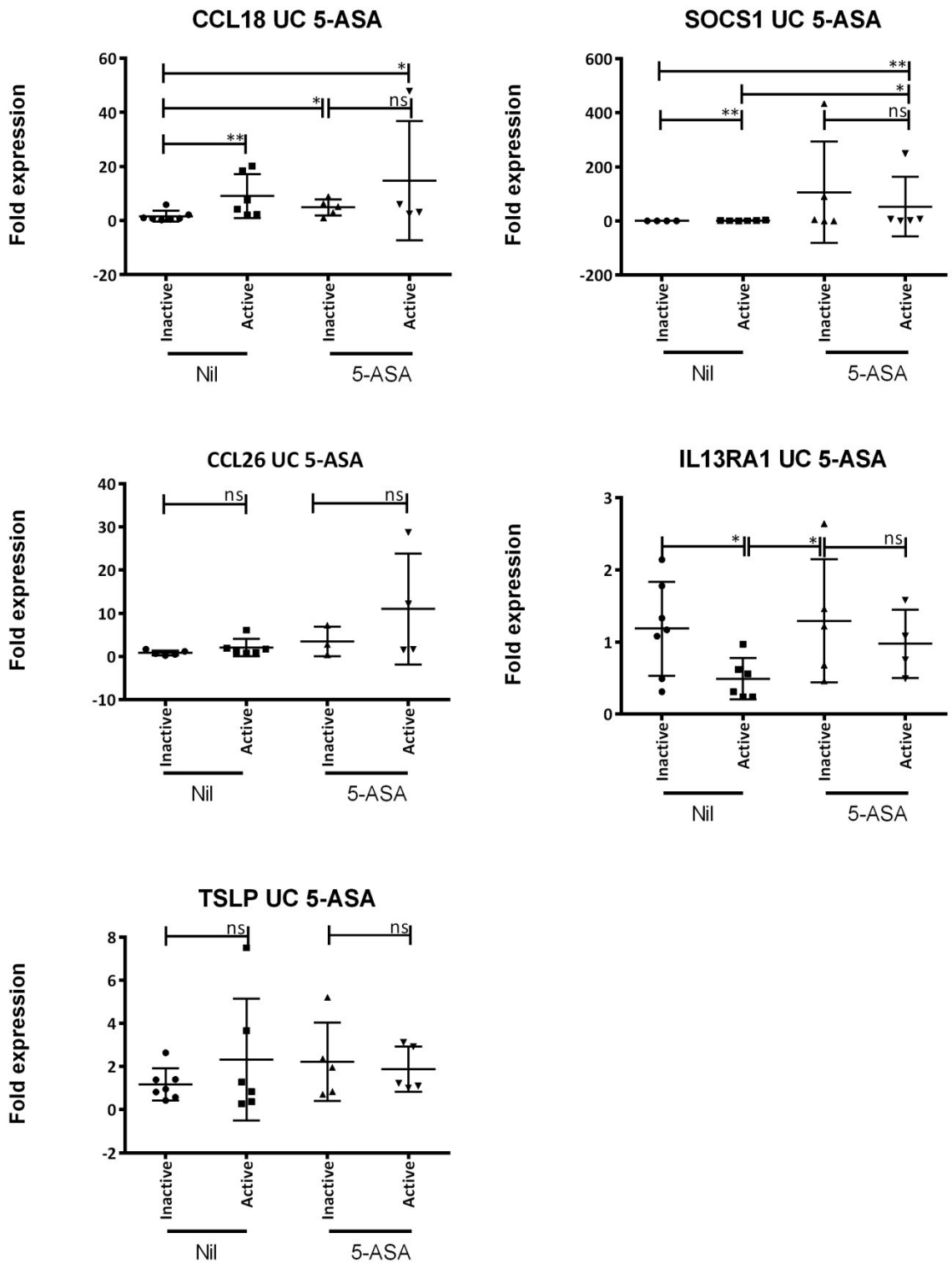


***Figure 63**. mRNA expression profile of Th2- related genes in active and unaffected, inactive UC samples from patients untreated (Nil) or undergoing 5-aminosalicylic acid (5-ASA) treatment. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *: P-value<0.05, ** P-value<0.01, *** P-value<0.001.*

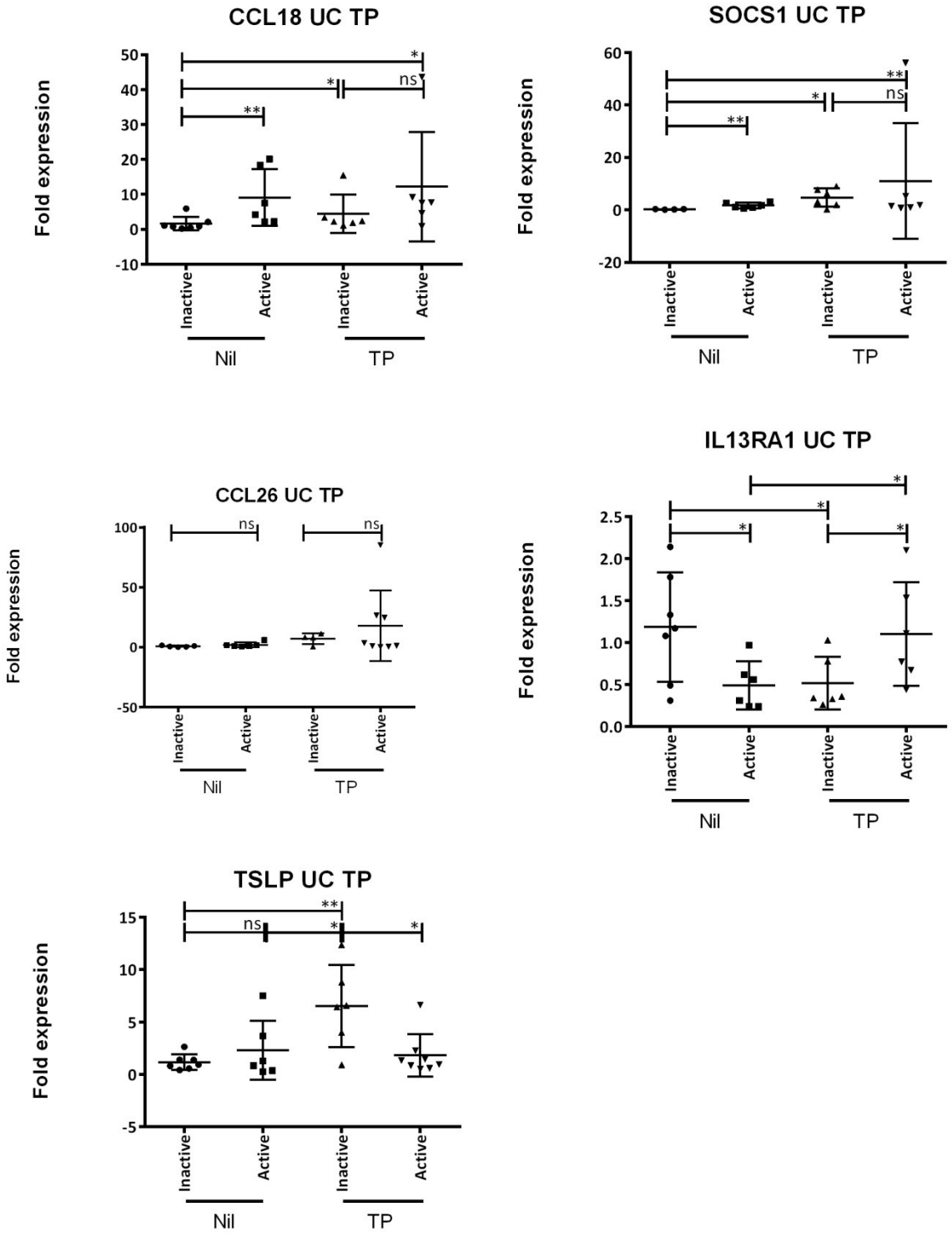


***Figure 64**. mRNA expression profile of Th2- related genes in active and unaffected, inactive UC samples from patients untreated (Nil) or undergoing Thiopurine (TP) treatment. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *: P-value<0.05, ** P-value<0.01, *** P-value<0.001.*

Genes involved in tissue remodelling and fibrosis (SERPINE1, MMP9, MMP12 and TGF-β1) showed a significant up-regulation in inflamed treatment naïve samples compared to unaffected, inactive untreated UC samples (***Figures 65*** and ***66***). COL1A2 was increased but did not reach significance.

In patients treated with 5 ASA and Thiopurines patients in remission had low levels of SERPINE1 comparable to the unaffected, inactive treatment naïve samples, but in the active treatment unresponsive group there was no significant reduction of SERPINE1. However, the significant difference observed in the treatment naïve group between unaffected, inactive and active samples was lost (***Figures 65 and 66***).

MMP9 and MMP12 levels were significantly increased in active treatment naïve patients as compared to unaffected, inactive controls. In the treatment group with 5-ASA, MMP9 and MMP12 levels were not significantly different when compared to unaffected, inactive treatment naïve patients, but remained significantly increased in patients not responding to 5-ASA (***Figure 65***). MMP9 and MMP12 levels in the Thiopurine group in patients not responding to treatment were persistently significantly up-regulated at levels higher than observed in active treatment naïve patients (***Figure 66***). There was no significant difference between unaffected, inactive treatment naïve and unaffected, inactive 5-ASA treated patients (***Figure 65***). A similar result was observed in the Thiopurine group where there was persistent high expression of MMP9 and MMP12 in the non-responders, but also a significantly elevated MMP9 in the unaffected, inactive treatment group with Thiopurines (***Figure 66***).

TGF-β1 was significantly increased in active treatment naïve patients compared to their unaffected, inactive controls. This significant increase was lost in the treatment groups with 5-ASA and Thiopurines comparing unaffected, inactive to active samples. Moreover, a reduction of TGF-β1 was demonstrated in both active UC samples treated with 5-ASA and Thiopurines, which reached significance in the Thiopurine group (***Figures 65*** and ***66***).

COL1A2 was not significantly affected by treatment with 5-ASA or Thiopurines (***Figures 65*** and ***66***), but an increase of CO1A2 in both treatment groups was noted, which reached significance in the active Thiopurine group in non-responders (***Figure 66***).

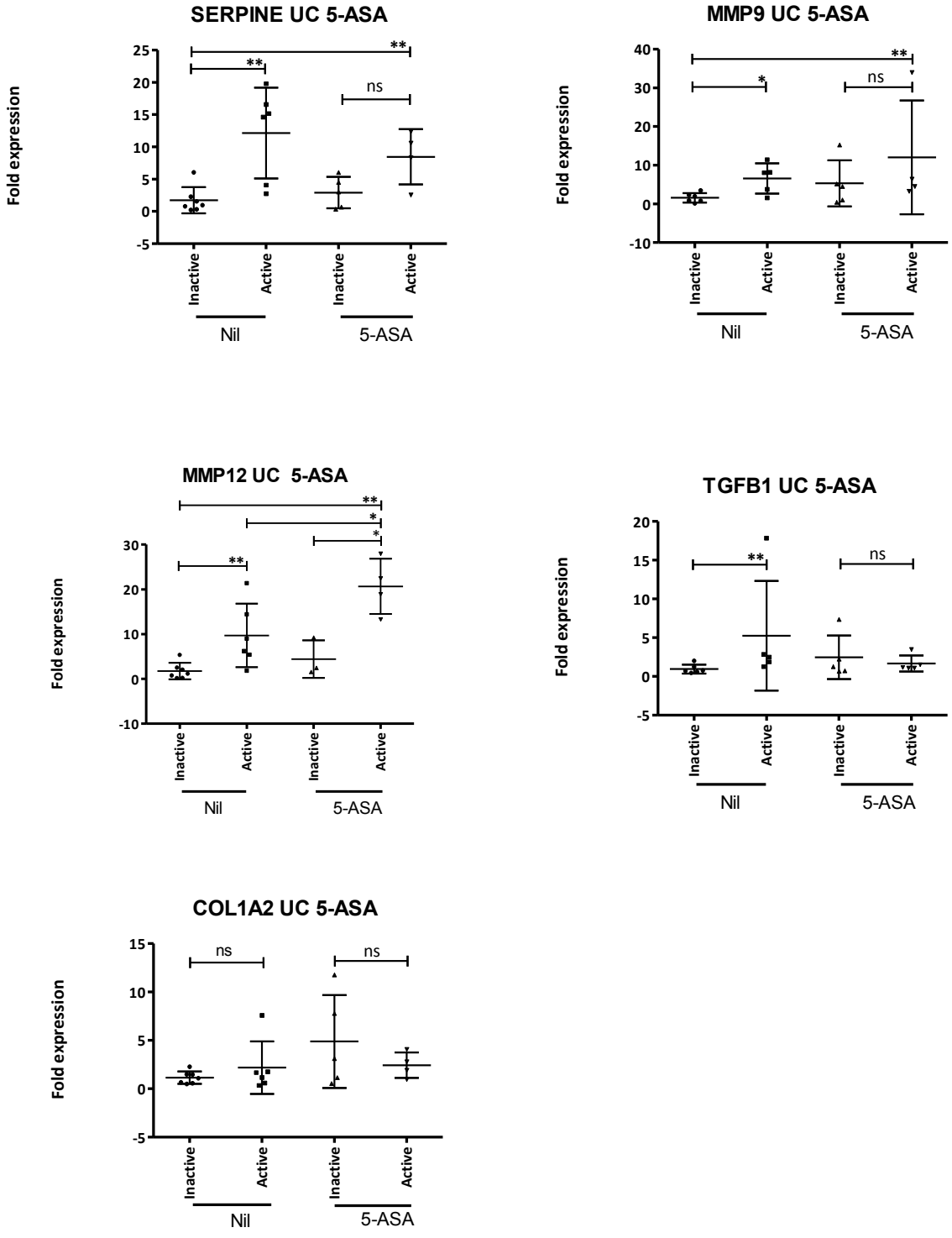


***Figure 65**. mRNA expression profile of fibrosis/remodelling- related genes in active and unaffected, inactive UC samples from patients untreated (Nil) or undergoing 5-aminosalicylic acid (5-ASA) treatment. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *: P-value<0.05, ** P-value<0.01, *** P-value<0.001.*

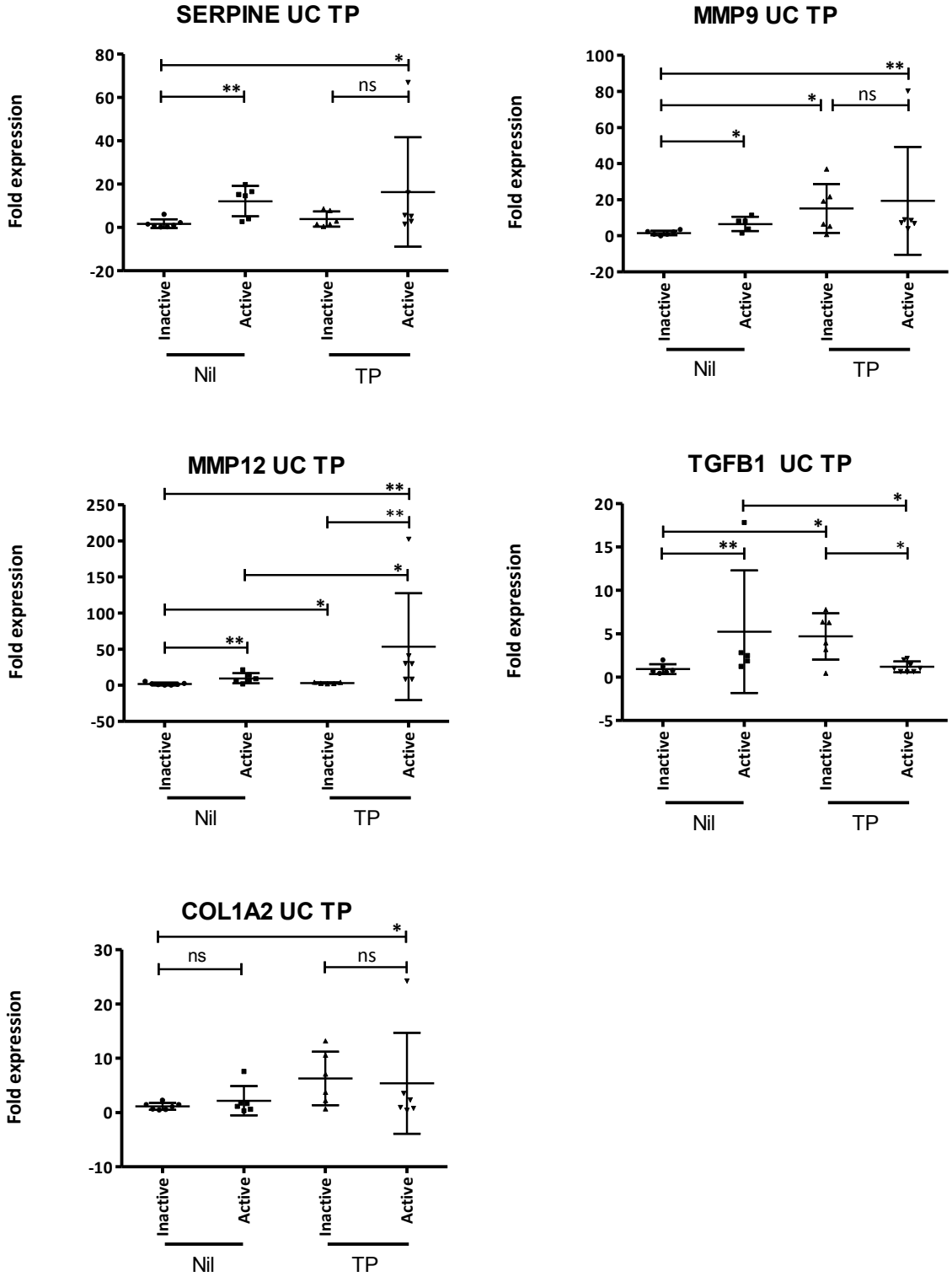


*Figure 66. mRNA expression profile of fibrosis/remodelling- related genes in active and unaffected, inactive UC samples from patients untreated (Nil) or undergoing Thiopurine (TP) treatment. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *: P-value<0.05, ** P-value<0.01, *** P-value<0.001*

7.2 MicroRNA and mRNA expression in Crohn's disease

To evaluate the immune profile of mucosal biopsies of patients with CD, samples depending on their inflammatory status were pooled according to "unaffected, inactive" or "active" inflammation regardless of current medical treatment (see demographic data, ***Table 12***). Pinch biopsies were collected from unaffected, inactive and active mucosa in the sigmoid colon from patients with CD (see sections ***2.7*** and ***2.8***). Patients with CD were graded according to the Simple Endoscopic Score for Crohn's Disease (SES-CD) and unaffected, inactive sigmoid samples had a score of 0, and active sigmoid biopsies had a score of greater or equal to 1. A total number of 46 sigmoid biopsies from CD patients (unaffected, inactive and active) were analysed. RNA was extracted using TRIzol (see ***section 2.16***) and assayed by RT-qPCR (see ***sections 2.17 and 2.18***). Samples which showed lower than twice the standard deviation of the Ct value of all the samples in housekeeping genes (RNU44 for microRNA and GAPDH for mRNA) were excluded as indicative of RNA degradation or poor quantity/quality. Samples with similar Ct values for the housekeepers (RNU44 and GAPDH) were included in the analysis. The results are shown in ***Figure 67*** for microRNA and ***Figure 68*** (Th1 genes), ***Figure 69*** (Th2 genes) and ***Figure 70*** (tissue remodelling/fibrosis genes) for mRNA. ***Table 18*** shows the number of biopsies taken into account for the analysis.

MicroRNA	Unaffected, inactive	Active
miR-31	19	18
miR-146a	19	18
miR-155	19	18

Table 18. Sample sizes for microRNA analysis in samples from patients with unaffected, inactive and active UC.

7.2.1 microRNAs miR-31, miR-146a and miR-155 are dysregulated in CD

MicroRNAs miR-31, miR-146a and miR-155 were all significantly up regulated in active colonic sigmoid CD compared to unaffected, inactive mucosa (***Figure 67***).

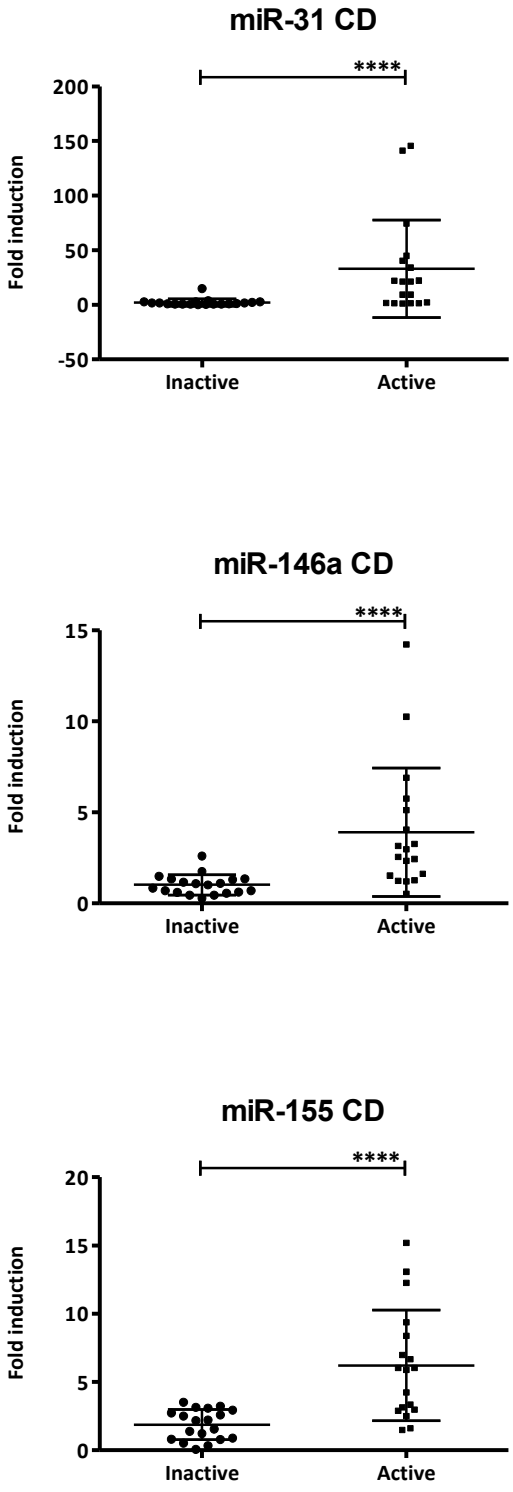


Figure 67. MiR-31, miR-146a and miR-155 expression in unaffected, inactive and active CD samples. MicroRNA expression was determined by RT-qPCR. Sample size was N=19 in unaffected, inactive and N=18 for active samples. Mann-Whitney t-test was performed. *= P-value<0.05, **= P-value<0.01, ***= P-value<0.001, ****= P-value<0.0001

7.2.2 mRNA expression profile of CD

After determining that miR-31, miR-155 and miR-146a were significantly up regulated in active CD samples (***Figure 67***), we analysed the mRNA expression profile of the same unaffected, inactive and active CD sigmoid biopsies. We assayed a panel of pro-inflammatory mediators subdivided into Th1 (TNF-α, IL1β, IL12βR1, IL12βR2 and IFN-g) and Th2 related genes (CCL18, SOCS1, CCL26, IL13Rα1 and TSLP) (***Figures 68*** and ***69,*** respectively) and also created a profile of genes related to fibrosis and tissue remodelling (SERPINE1, MMP9, MMP12, TGF-β1 and COL1A2) (***Figure 70***).

In Th1-related genes (***Figure 68***), the pro-inflammatory mediators TNF-α, IL1β and IFN-γ were found significantly up-regulated in active samples compared to unaffected, inactive samples. Both IL12Rβ1 and IL12Rβ2 were also significantly up regulated in active colonic CD (***Figure 68***).

In Th2-related genes (***Figure 69***), CCL18, SOCS1 and CCL26 were found significantly over expressed in active CD compared to unaffected, inactive mucosal biopsies. IL13Rα1 was significantly down-regulated in active mucosal inflammation compared to unaffected, inactive mucosa. TSLP was not significantly differentially expressed in unaffected, inactive and active CD.

The tissue remodelling and fibrosis gene demonstrated significant mRNA up-regulation of all genes tested (SERPINE1, MMP9, MMP12, TGF-β1 and COL1A2) (***Figure 70***).

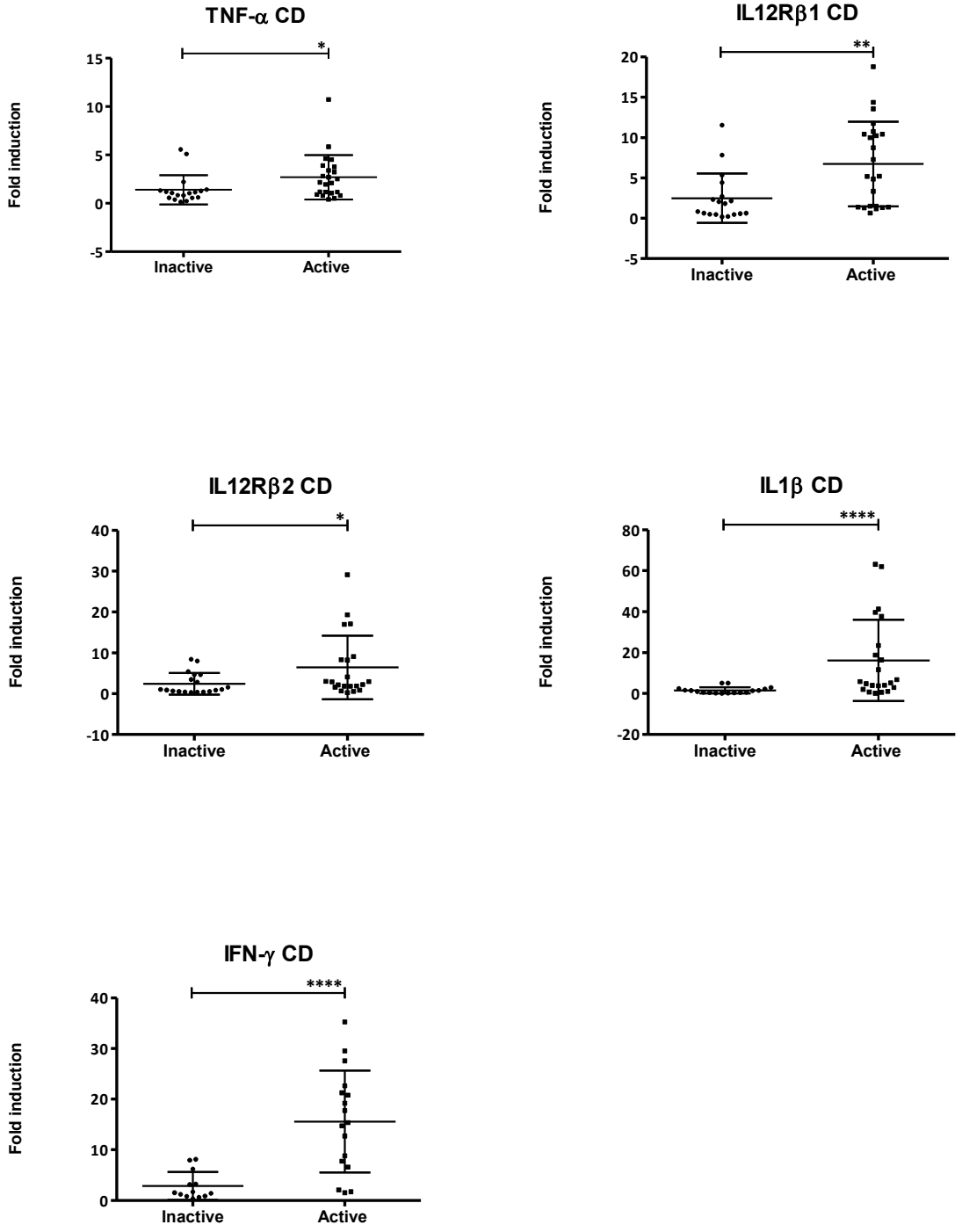


Figure 68. Th1 genes analysed in active and unaffected, inactive CD samples. mRNA expression was determined by RT-qPCR and fold induction calculated using GAPDH as housekeeper. N=18 (unaffected, inactive) and N=22 (active). Mann-Whitney t-test was performed.*= P-value<0.05, **= P-value<0.01, ***= P-value<0.001, ****= P-value<0.0001

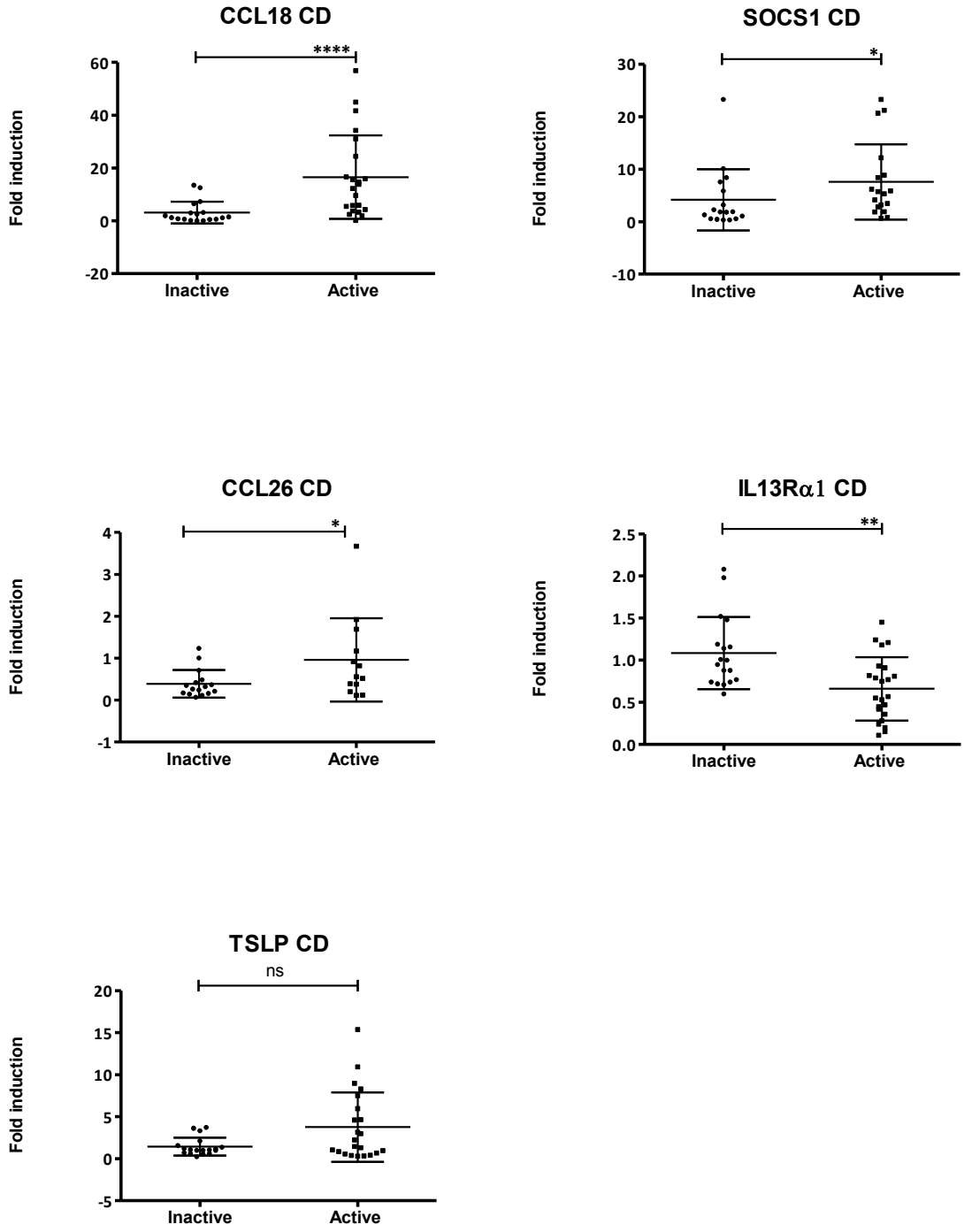


Figure 69. Th2 genes analysed in active and unaffected, inactive CD samples. mRNA expression was determined by RT-qPCR and fold induction calculated using GAPDH as housekeeper. N=18 (unaffected, inactive) and N=22. Mann-Whitney t-test was performed *= P-value<0.05, **= P-value<0.01, ***= P-value<0.001, ****= P-value<0.0001

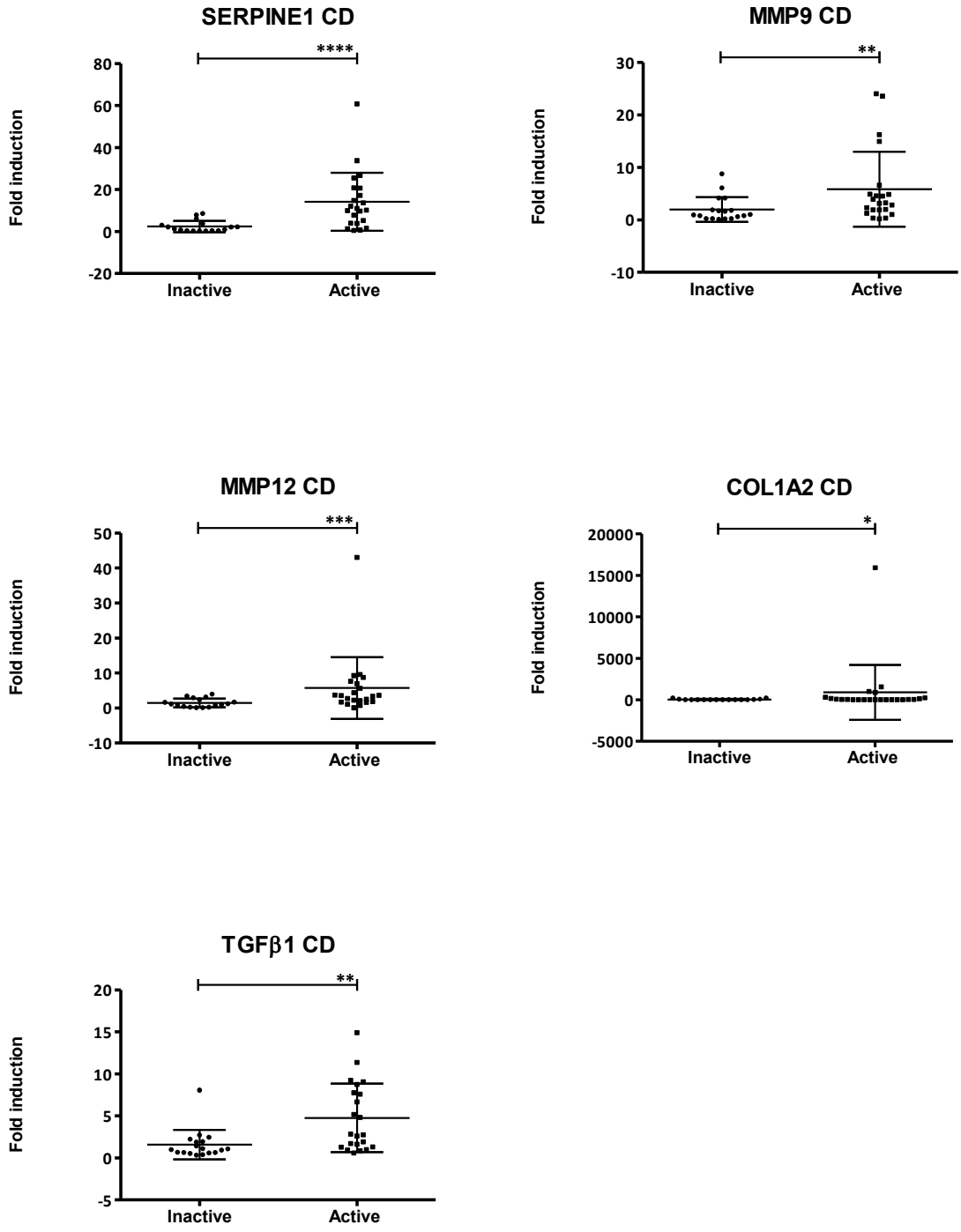


Figure 70. Fibrosis/remodelling genes analysed in active and unaffected, inactive CD samples. mRNA expression was determined by RT-qPCR and fold induction calculated using GAPDH as housekeeper. N=18 (unaffected, inactive) and N=22. Mann-Whitney t-test was performed *= P-value<0.05, **= P-value<0.01, ***= P-value<0.001, ****= P-value<0.0001.

7.2.3 mRNA and microRNA expression in CD is altered by Thiopurine and anti-TNF-α therapy

7.2.3.1 Introduction

To investigate the impact of medical treatments on the microRNA and inflammatory/tissue remodelling mRNA expression profile of CD patients, we subdivided the samples from unaffected, inactive and active CD tissue and grouped them according to their drug treatment, Thiopurines (TP) or anti-TNF-α agents, respectively. Each group contained a similar number of samples (sample sizes are indicated in ***Table 12***) and fold induction was calculated comparing to treatment naïve unaffected, inactive CD. RNA was extracted using TRIzol (see ***section 2.16***) and analysed by RT-qPCR (see ***sections 2.17 and 2.18***). MicroRNAs miR-31, miR-146a and miR-155 expression was assayed (***Figure 71***). Messenger RNA expression profiles are shown in ***Figures 72*** to ***77***. Within each medication group, genes were sub-grouped according to their general function: Th1 (***Figures 72 and 73*** for TP and anti-TNF-α, respectively), Th2 (***Figures 74 and 75*** for TP and anti-TNF-α, respectively) and fibrosis/tissue remodelling (***Figures 76 and 77*** for TP anti-TNF-α, respectively).

7.2.3.2 Expression of miR-31, miR-146a and miR-155 is modulated by Thiopurine and anti-TNF-α treatment in Crohn's disease.

In order to determine if drug treatment affected the colonic microRNA profile of Crohn's patients, we analysed the expression of miR-31, miR-155 and miR-146 by RT-qPCR (see ***sections 2.16, 2.17 and 2.18***). We assayed unaffected, inactive and active samples from treatment naïve patients with sigmoid CD (Nil), and likewise undergoing treatment with either Thiopurines or anti-TNF-α compounds. Sample size is indicated in ***Table 19***. ***Figure 71*** shows the results.

Nil		**TP**		**Anti-TNF-α**	
Unaffected, inactive	Active	Unaffected, inactive	Active	Unaffected, inactive	Active
6	8	5	5	6	7

Table 19. Sample sizes for microRNA expression analysis in Crohn's patients untreated and undergoing AZA or anti-TNF-α.

MiR-31, miR-146a and miR-155 were found to be significantly up-regulated in active sigmoid biopsies compared to unaffected, inactive samples from treatment naïve patients (***Figure 72***).

Expression profiles of miR-31, miR-146a and miR-155 varied considerably according to medication (Thiopurines or anti-TNFα compounds) patients were treated with. Thiopurines abolished the significant difference seen in treatment naïve patients in all microRNAs assayed (***Figure 71***).

MiR-31 interestingly was significantly increased in treatment responders compared to active treatment naïve patients and the levels were higher than in non-responders to Thiopurines. MiR-31 was also raised in responders at a higher level than in non-responders compared to treatment naïve unaffected, inactive samples. MiR-146a was significantly less expressed in responders to Thiopurines compared to active treatment naïve patients, but still showed much higher levels compared to unaffected, inactive treatment naïve samples. Levels of miR-146a in non-responders to Thiopurines showed significantly lower expression compared to treatment naïve active samples and was also expressed less compared to treatment responders, but did not reach significance. MiR-155 expression in the Thiopurine group behaved similar to miR-31, showing a significant increase of miR-155 in treatment responders compared to active treatment naïve patients. In non-responders to Thiopurines miR-155 levels were significantly lower than in active treatment naïve patients (***Figure 71***).

Results in the anti-TNF-α treatment group for microRNAs miR-31, miR-146a and miR-155 varied significantly from results obtained in the Thiopurine group. MiR-31 and miR-155 levels in treatment responders were comparable to treatment naïve patients. miR-146 levels in treatment responders were significantly lower in responders to anti-TNF-α treatment. In patients who did not respond to treatment miR-31, miR-146a and miR-155 expression remained significantly raised compared to treatment responders. In the case of miR-31 and miR-155 the levels in the non-responders group were significantly up-regulated compared to all other groups. MiR-146a in treatment resistant patients, although significantly lower than in active treatment naïve patients, remained significantly more expressed as compared to treatment naïve unaffected, inactive sigmoid biopsy samples (***Figure 71***).

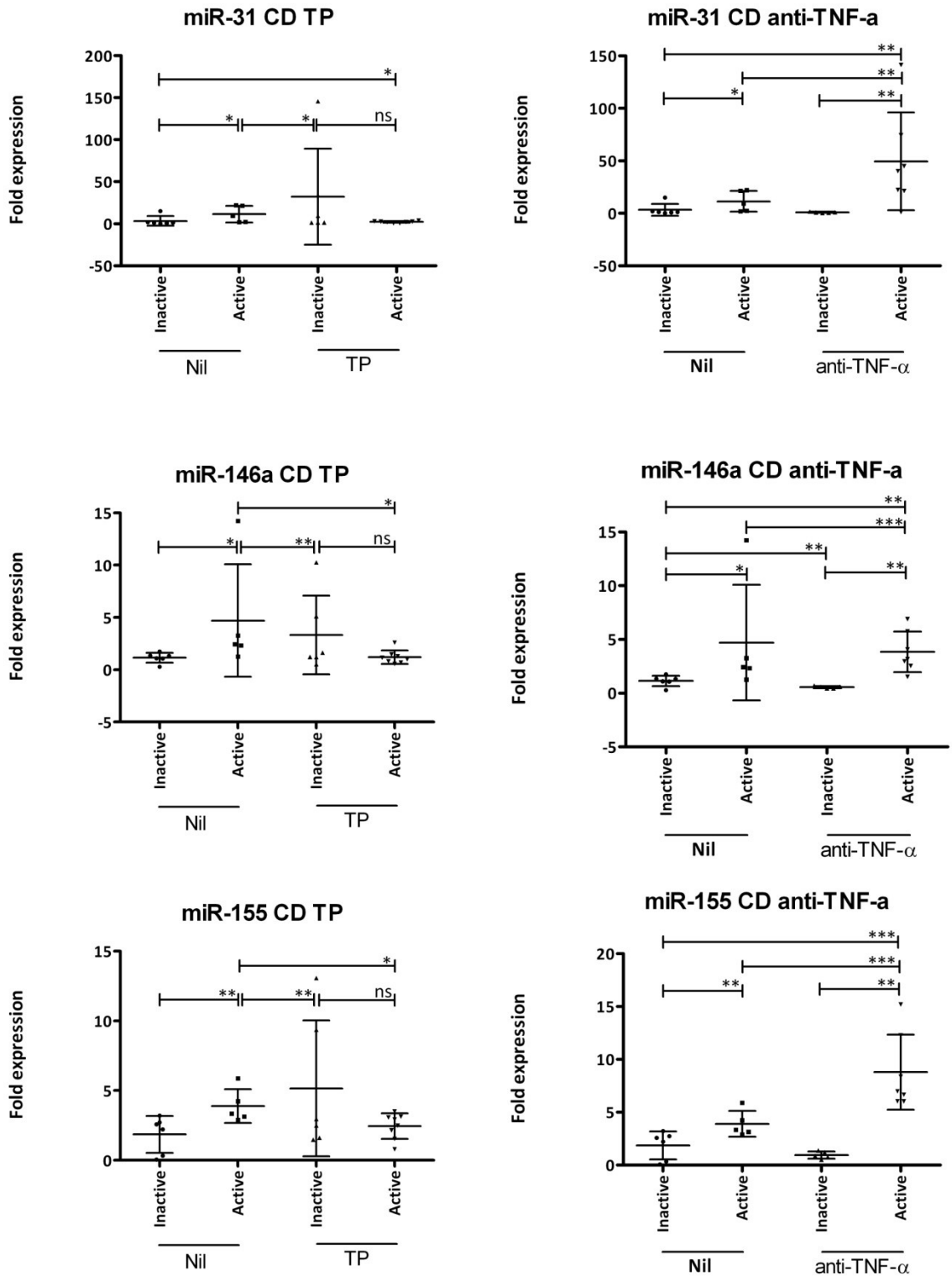


Figure 71. MiR-31, miR-155 and miR-146a expression in active and unaffected, inactive sigmoid CD samples from treatment naïve patients or patients undergoing treatment with TP or anti-TNF-α. MicroRNA expression was determined by RT-qPCR. Mann-Whitney t-test was performed. *= P-value<0.05, **= P-value<0.01, ***= P-value<0.001.

7.2.3.3 Crohn's mRNA expression is modulated by AZA and Infliximab treatments

After determining that Thiopurines and anti-TNF-α compounds significantly modulate the levels of microRNAs miR-31, miR-146a and miR-155, we analysed the impact of these treatments on the mRNA levels of genes involved in the inflammatory and immune response and their effect on fibrosis and tissue remodelling genes.

Samples were divided according to medication (TP or anti-TNF-α). Th1, Th2 and fibrosis/remodelling gene expression was analysed. Results are shown in F***igure 72*** (Th1 TP), ***Figure 73*** (Th1 anti-TNF-α), ***Figure 74*** (Th2 TP), ***Figure 75*** (Th2 anti-TNF-α), ***Figure 76*** (fibrosis/remodelling TP) and ***Figure77*** (fibrosis/remodelling anti-TNF-α), respectively.

Sample sizes are indicated in ***Table 20***.

Nil		**TP**		**anti-TNF-α**	
Unaffected, inactive	Active	Unaffected, inactive	Active	Unaffected, inactive	Active
6	8	5	5	6	7

Table 20. Sample sizes for mRNA expression analysis in Crohn's disease patients. Treatment naïve or undergoing treatment with TP or anti-TNF-α.

Figures 73 and ***74*** demonstrate the mRNA expression of Th1-related genes TNF-α, IL1β, IL12βR1, IL12βR2 and INF-γ. In treatment naïve patients all genes, except for IL12βR2, were significantly up-regulated in active sigmoid CD (***Figures 72 and 73***). In the treatment groups with Thiopurines and anti-TNF-α compounds the significant difference between unaffected, inactive and active sigmoid CD was abolished for TNF-α, IL1β, IL12βR1 and IFN- γ mRNA expression. Both TP and anti-TNF-α compounds were able to effectively down-regulate the expression of TNF- α, IL12RB1, IL1B and IFN-γ in treatment responders compared to active treatment naïve patients (***Figures 72*** and ***73***). Patients, who did not respond to TP treatment, showed persistently raised mRNA levels of TNF-α, IL1β, IL12βR1, IL12βR2 and IFN-γ (***Figure 72***). In the anti-TNF-α group a similar result was observed with persistent up-regulation of TNF-α, IL1β, IL12βR1, IL12βR2 and IFN-γ. This reached significance in the case of IL12βR1, IL12βR2 and IFN-γ compared to treatment naïve and anti-TNF-α treated unaffected, inactive sigmoid biopsies (***Figure 73***).

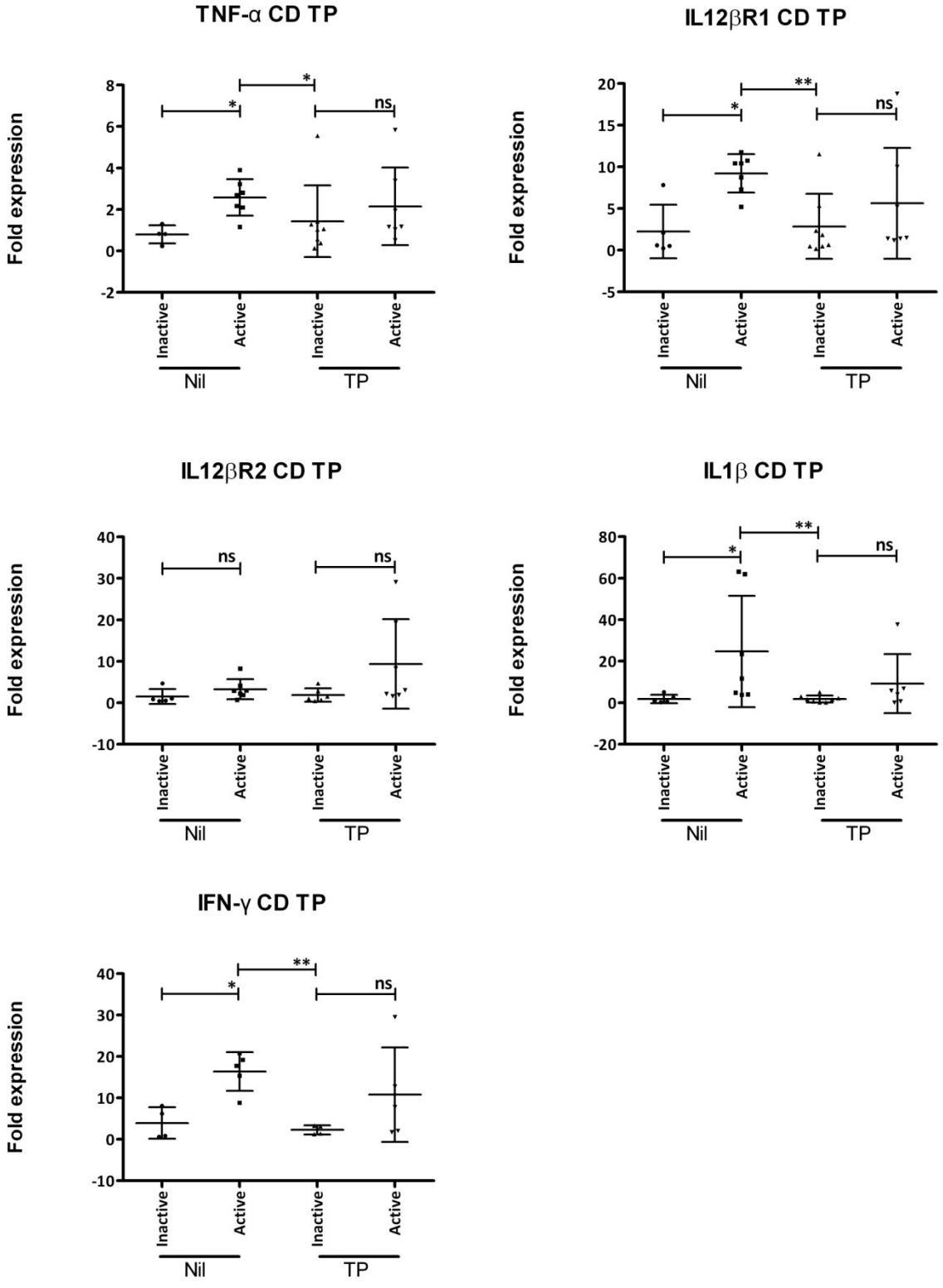


Figure 72. mRNA expression in active and unaffected, inactive sigmoid CD samples from treatment naïve patients or patients undergoing treatment with TP.mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *=P-value<0.05, **= P-value<0.01, ***= P-value<0.001.

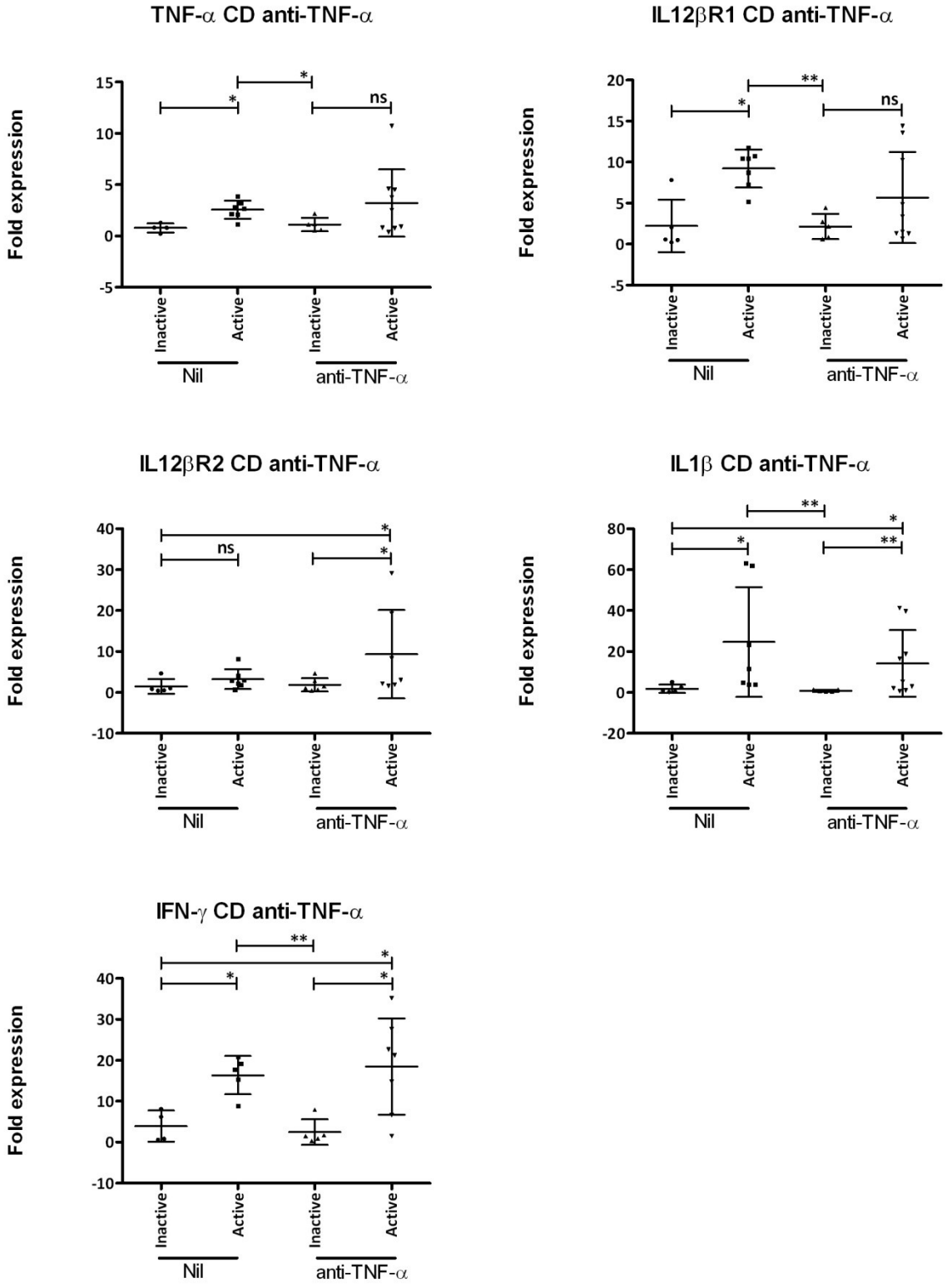


Figure 73. mRNA expression in active and unaffected, inactive sigmoid CD samples from treatment naïve patients or patients undergoing treatment with anti-TNF-α. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *=P-value<0.05, **= P-value<0.01, ***= P-value<0.001.

Th2-related genes were analyzed for the expression of CCL18, SOCS1, CCL26, IL13Rα1 and TSLP. In treatment naïve patients, CCL18, SOCS1, CCl26 and TSLP showed significant up-regulation in active sigmoid biopsies in CD compared to unaffected, inactive mucosa in the sigmoid colon in CD, whereas IL13Rα1 was found significantly reduced in active disease similar to results in active UC (***Figures 74*** and ***75***). Both Thiopurines and anti-TNF-α treatment abolished the significant difference between unaffected, inactive and active CD samples, apart from CCL18 in the anti-TNF-α group where the difference was less significant as observed in treatment naïve patients. In the Thiopurine group CCL18 and TSLP were significantly down-regulated and IL13Rα1 was significantly up-regulated in treatment responders (***Figure 74).*** Anti-TNF-α treatment effectively down-regulated CCL18, SOCS1, CCL26 and TSLP, and up-regulated IL13Rα1 in treatment responders, all of which reached significance compared to active treatment naïve patients (***Figures 75).*** Patients who did not respond to either Thiopurines or anti-TNF-α treatment generally had higher levels of CCL18, SOCS1, CCL26 and TSLP as compared to patients in remission and unaffected, inactive treatment naïve patients. IL13Rα1 was significantly elevated in non-responders compared to active treatment naïve patients in both medication groups (***Figures 74*** and ***75***). In the case of TSLP, a Th2- master regulator, both Thiopurines and anti-TNF-α treatment showed to significantly reduce its levels in active disease (***Figures 74*** and ***75***).

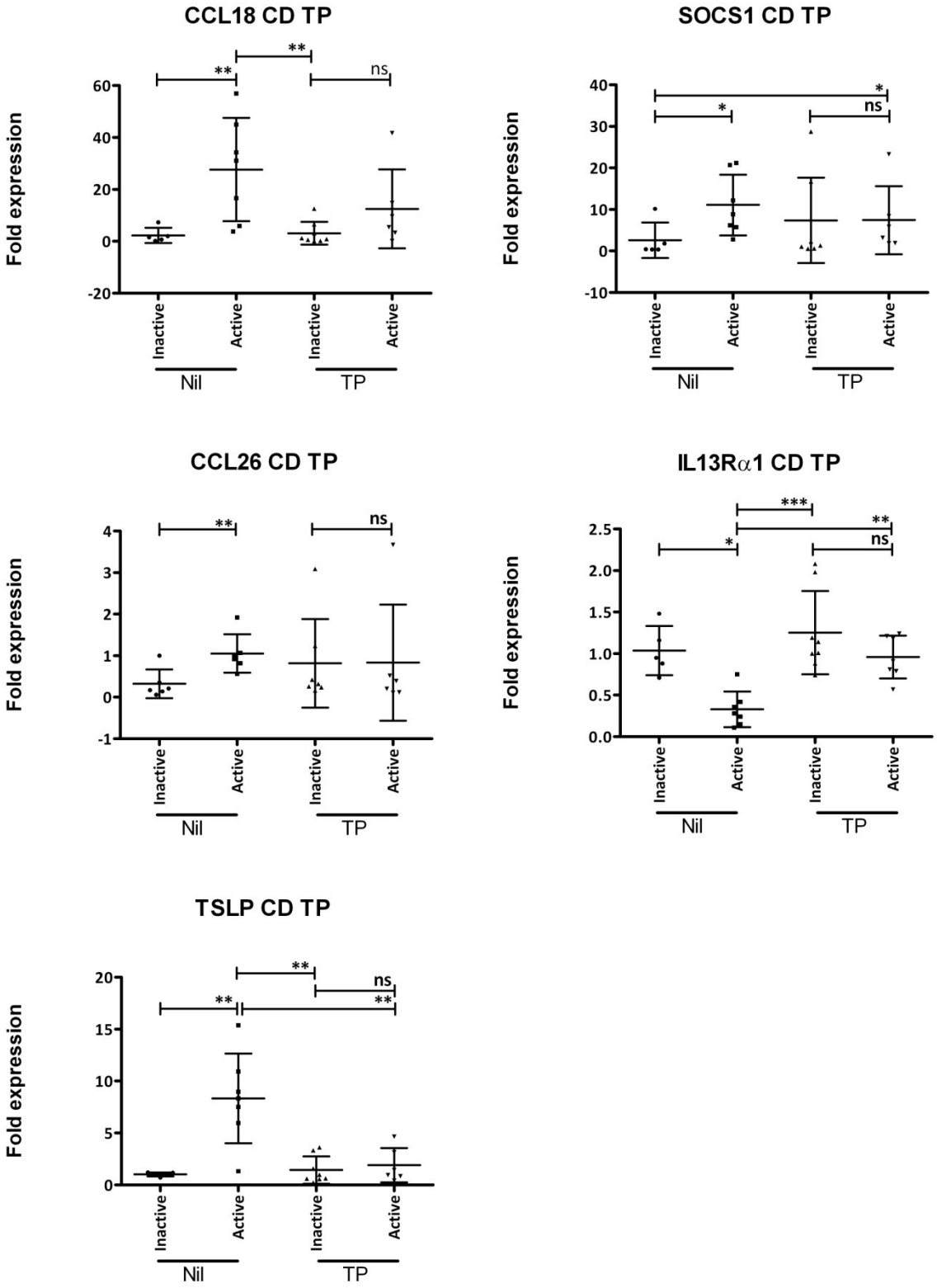


Figure 74. mRNA expression in active and unaffected, inactive sigmoid CD samples from treatment naïve patients or patients undergoing treatment with Thiopurines (TP). mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *=P-value<0.05, **= P-value<0.01, ***= P-value<0.001.

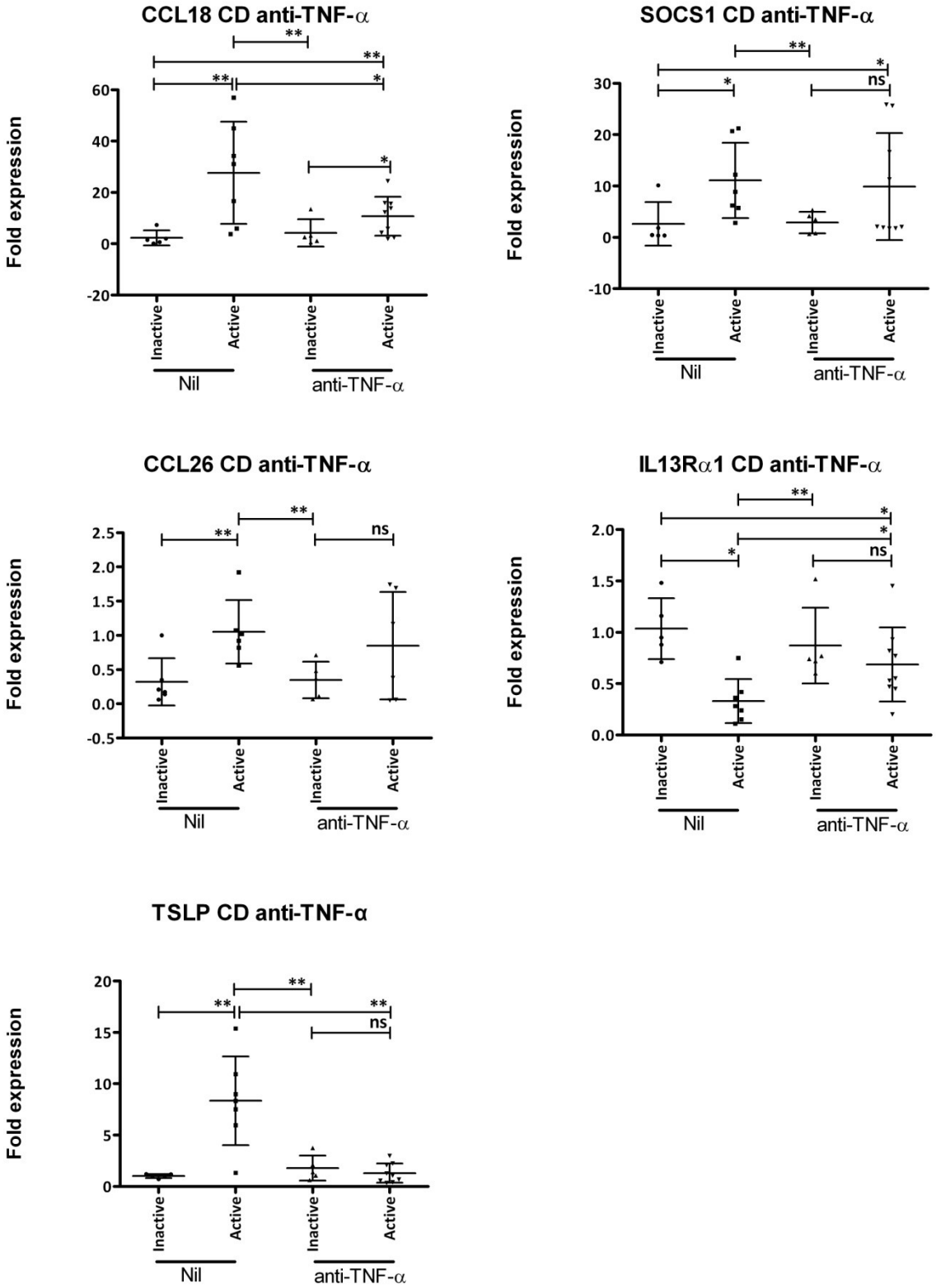


Figure 75. mRNA expression in active and unaffected, inactive sigmoid CD samples from treatment naïve patients or patients undergoing treatment with anti-TNF-α. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *=P-value<0.05, **= P-value<0.01, ***= P-value<0.001.

With regards to the fibrotic/remodelling panel of genes, we analysed the mRNA expression of SERPINE1, MMP9, MMP12, COL1A2 and TGF-β1. ***Figures 77*** and ***78*** show the impact of Thiopurines and anti-TNF-α treatments on the expression of genes involved in tissue remodelling and fibrosis.

SERPINE1, COL1A2 and TGF-β1 were significantly increased in samples of active treatment naïve patients compared to unaffected, inactive treatment naïve CD. MMP9 and MMP12 did not demonstrate a significant difference between unaffected, inactive and active treatment naïve patients (***Figures 76*** and ***77)***.

SERPINE1, COL1A2 and TGF-β were significantly down-regulated in treatment responders in the Thiopurine group compared to active treatment naïve patients. In patients who did not respond to treatment SERPINE1 remained significantly up-regulated in non-responders. COL1A2 was elevated in responders and non-responders in the Thiopurine group, but at much lower levels compared to active treatment naïve patients. TGF-β1 levels were similar in responders and non-responders in the Thiopurine group, but at much significantly lower levels as compared to active treatment naïve patients. MMP9 and MMP12 levels were higher in non-responders in the Thiopourine group but did not reach significance (***Figure 76).***

In the anti-TNF-α treatment group responders showed significantly lower expression of SERPINE1, MMP9, MMP12, COL1A2 and TGF-β1 when compared to active treatment naïve patients. In non-responders persistently high levels of SERPINE1, MMP12, COL1A2 and TGF-β1 were observed when compared to treatment naïve patients. MMP9 and MMP12 were significantly raised in non-responders to anti-TNF-α treatment as compared to patients in remission. The significant difference in the expression of SERPINE1, COL1A2 and TGF-β observed in the treatment naïve group between unaffected, inactive and active CD was abolished in the anti-TNF-α treatment group (***Figures 77).***

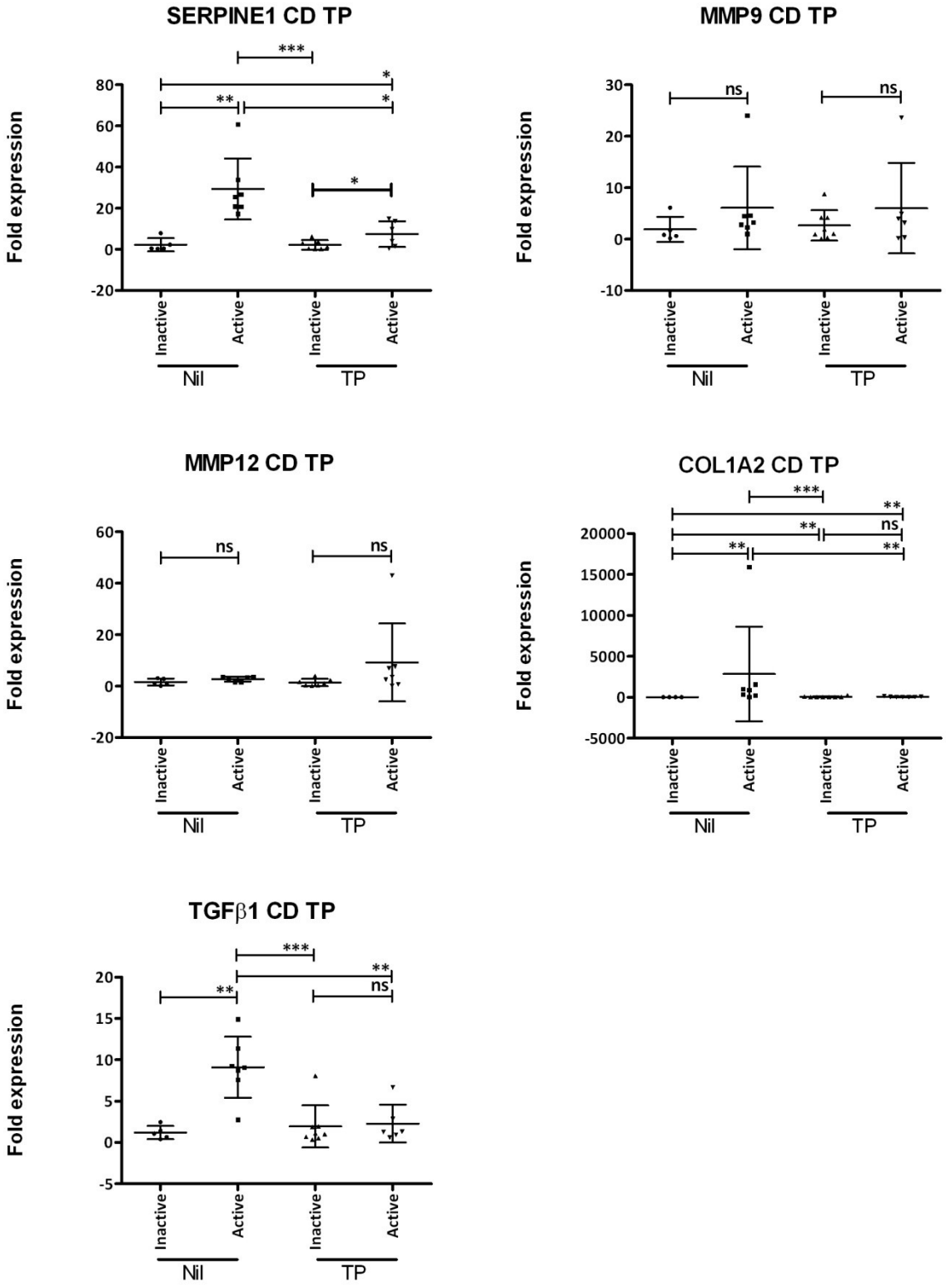


Figure 76. mRNA expression profile of fibrosis/remodelling genes in active and unaffected, inactive Crohn's samples from treatment naïve patients (Nil) or undergoing Thiopurine treatment. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *: P-value<0.05, ** P-value<0.01, *** P-value<0.001.

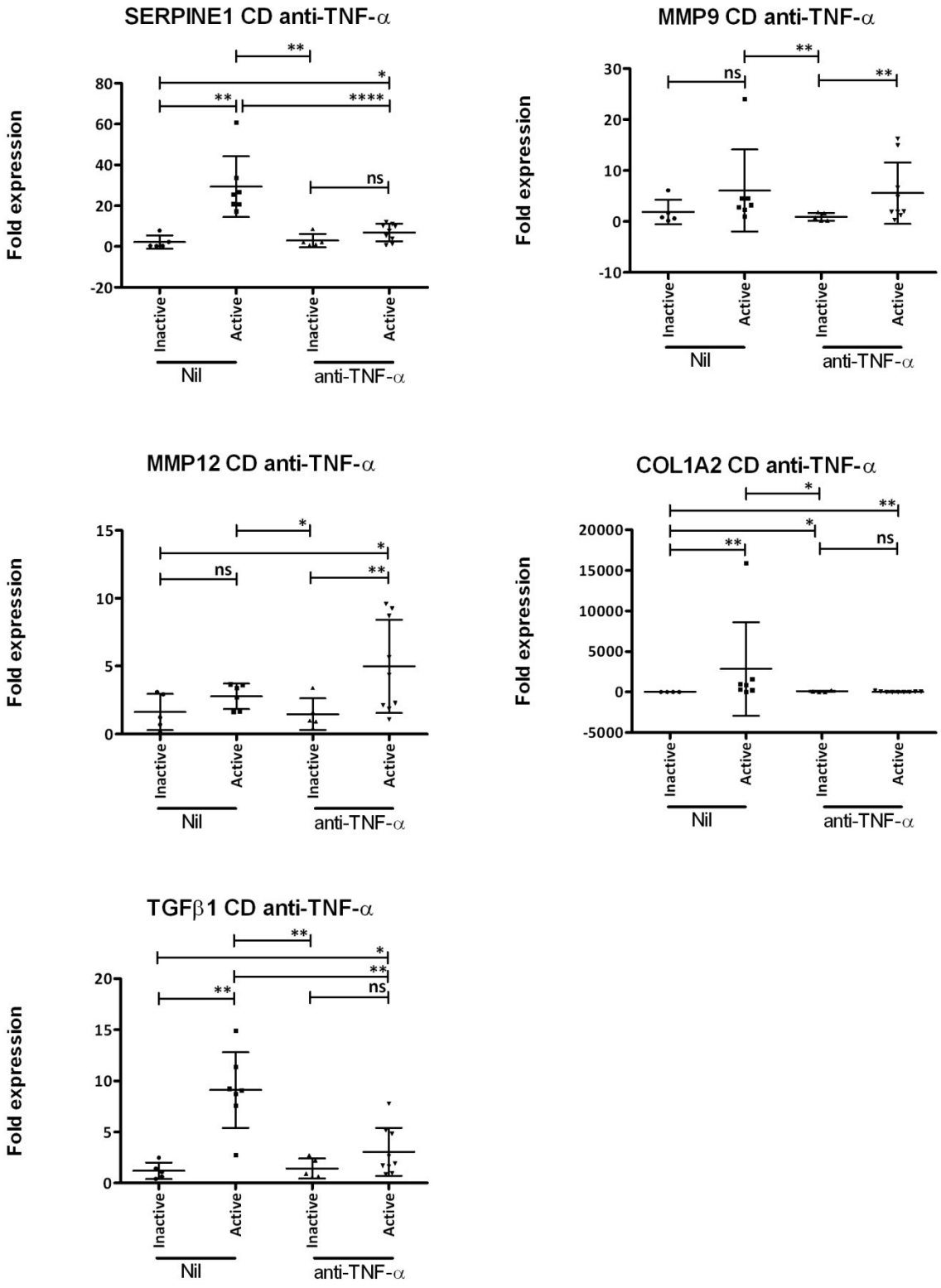


Figure 77.mRNA expression profile of fibrosis/remodelling genes in active and unaffected, inactive Crohn's samples from treatment naïve patients (Nil) or undergoing anti-TNF-α treatment. mRNA expression was determined by RT-qPCR and normalized against GAPDH. Mann-Whitney t-test was performed. *: P-value<0.05, ** P-value<0.01, *** P-value<0.001.Discussion

8 General Discussion

This project investigates the effects of microRNAs miR-31 and miR-155 on the IL-13 pathway by direct targeting IL13Rα1 in patients with UC. For the ease of reading and explaining of the results, discussion of the general chapters (*in vitro* work and UC/CD profiling and effects of therapy) will be done in subsections. The overall aim of this work was to determine the roles of miR-31 and miR-155 in the IL-13 pathway in UC via the direct targeting of IL13Rα1, which has been demonstrated in a series of *in vitro* experiments (***Chapters 4*** to ***6***) including microRNA transfection of biopsies (showing the clinical potential of our results), as well as mRNA and microRNA profiling of UC and CD patients showing the effect of medication in these (***Chapter 7***).

8.1 General discussion 1: MicroRNAs 31 and 155 down regulate the IL-13 pathway in UC

UC is an inflammatory disease of the colon affecting the mucosa and sub-mucosa of the large bowel. It is thought to be driven by a Th2-like response in which NKT cells producing IL-13 (and IL-5) is the major factor, both IL-13 and NKT cells causing direct toxic effects to the mucosal surface of the colon (Strober and Fuss 2011). In the healthy gut mucosa, luminal antigens and bacterial flora are sampled by the innate immune system mediated by TLRs determining as to whether to tolerate a foreign antigen or eliminate it. This balance is lost in UC due to the disruption of the mucosal barrier by raised levels of IL-13 released by high numbers of NKT cells, which phosphorylates and activates STAT6 via IL13Rα1 (Fuss, Heller et al. 2004). Increased STAT6 phosphorylation has been associated with UC and its inhibition with siRNAs or HDAC inhibitors has been shown to reduce the toxic effects of IL-13 on epithelial cells (Rosen, Frey et al. 2011). SOCS1 also has been shown to influence the level of inflammation by down-regulating STAT6 and being induced by Interferon-β-1a in patients with UC (Mannon, Hornung et al. 2011).

Dysregulation of microRNAs in UC has been reported in the literature and both miR-31 and miR-155 are well documented to be increased in inflamed UC tissue (Fasseu, Treton et al. 2010, Takagi, Naito et al. 2010, Olaru, Selaru et al. 2011). MiR-31 has been predicated by several microRNA bioinformatics tools (TargetScan, Microrna.org, PITA algorithm) to target IL13Rα1 and miR-155 has been shown to reduce the expression of IL13Rα1 in human macrophages by our group before (Martinez-Nunez, Louafi et al. 2011).

Our patient groups consisted of eleven normal colonic tissue samples from healthy patients undergoing colonoscopy for polyp surveillance, and eleven samples from patients suffering from UC affecting the distal colon. UC patients provided unaffected inactive UC (endoscopic Mayo score 0-1) tissue with matched paired inflamed (endoscopic Mayo score 2-3) UC tissue from the same patient in the proximity of 10cm, representing the perfect control for each inflamed sample. Age, sex, duration of disease, disease activity and medication of the UC cohort was noted and was representative of the general UC population (***Table 7***). We also assayed IL13Rα1 protein expression in normal controls, unaffected inactive and active UC samples. Epithelial cells isolated from biopsies of normal controls, unaffected, inactive and active UC were also analysed

and provided further data investigating the dysregulation of the IL-13/STAT6 pathway in ulcerative colitis and the role of microRNAs miR-31 and miR-155.

In vitro experiments were carried out in HT-29 cells, an epithelial colonic cell line, THP-1 cells and monocyte derived human macrophages, providing a model for macrophages and dendritic cells of the innate immune system of the sub-mucosa. The experimental approach aimed to investigate the effect of miR-31 and miR-155 and their combination on the IL13Rα1 and on the IL-13 pathway via STAT6 activation in cellular models mimicking the gut environment. To close the cycle from patient to *in vitro* models and back, we took our observations and findings patient samples and in *in vitro* cell models back to patient samples, testing the hypothesis in a clinically centred ex vivo model on the impact of miR-31 and miR-155 on the IL-13/STAT6 pathway in active UC.

In our data the mRNA expression of IL-13 dependent genes in patients with active UC (endoscopic Mayo score >2) showed a significant up-regulation of CCL18, a novel finding in UC, SOCS1, CCL26, SERPINE1 and MMP9 consistent with the increased IL-13 activity reported in the literature (Ott, Musfeldt et al. 2004, Backhed, Ley et al. 2005, Manichanh, Rigottier-Gois et al. 2006, Strober and Fuss 2011), (Blanchard, Durual et al. 2005, Manousou, Kolios et al. 2010) (see ***Figure 13***).

Because CCL18 induces the release of chemokines attracting lymphocytes, dendritic cells and monocytes to the site of inflammation, and is regulated by the Th2 cytokines IL-4 and IL-13 and by IL-10 (Meau-Petit, Tasseau et al. 2010, Strulovici-Barel, Omberg et al. 2010) we considered it was an important cytokine to be tested. We were able to demonstrate its significant up-regulation in inflamed UC and a relative down-regulation in unaffected, inactive disease as compared to normal samples (***Figure 13***), a novel finding not yet described in the literature.

SOCS1 was also significantly raised in our data in patients with active UC (***Figure 13***). SOCS1 is an inhibitor of STAT6 activation and shows a quick response to IL-13 stimulation, with a peak of mRNA expression one hour post-IL-3 stimulation in epithelial cells (Hebenstreit, Luft et al. 2003). This rapid increase could represent a first line of defence against IL-13 induced STAT6 activation counterbalancing the detrimental effect of IL-13 to the mucosa. As previously discussed, it has been shown that increased levels of SOCS1 induced by Interferon-β-1a therapy in patients with

UC produces an anti-inflammatory response (Mannon, Hornung et al. 2011). Considering that miR-155 directly targets SOCS1 (Wang, Hou et al. 2010) and previous work in our group has shown increased SOCS1 expression by transfection of anti-miR-155 in human macrophages (Martinez-Nunez, Louafi et al. 2011), all these findings certainly make SOCS1 an interesting prospect as a potential therapeutic target in UC.

SOCS1 mRNA has been significantly raised in our inflamed UC patient samples as compared to normal tissue and unaffected inactive UC (***Figure 13***). Interestingly, the levels of SOCS1 were reduced in unaffected inactive UC as compared to normal tissue; considering that one of its functions is to inhibit STAT6. This may reflect that unaffected, inactive UC tissue is more susceptible to STAT6 activation due to its lack of SOCS1 and therefore tissue inflammation.

High levels of SERPINE1, shown to be significantly up-regulated in our data in inflamed UC (***Figure 13***), have been demonstrated to impair wound healing via blocking smooth muscle migration via IL13Rα1 (Holubar and Harvey-Banchik 2007) as previously pointed out.

CCL26 was significantly increased in active disease as compared to normal controls (***Figure 13***) and this is in keeping with previously published work defining its role in the IL-13/STAT6 pathway and its increased expression in active UC (Blanchard, Durual et al. 2005, Manousou, Kolios et al. 2010).

The increased expression of MMP9 and its effect on the degradation of the ECM is well documented in active UC (Harvey and Sloviter 2005, Harvey, Bothma et al. 2005). Increased expression of MMP9 in active UC was confirmed by our results (***Figure 13***) and has also been shown to hinder wound healing by degrading the ECM (Harvey and Sloviter 2005, Harvey, Bothma et al. 2005). These pathological effects could possibly be positively influenced by down-regulating the IL13Rα1 with miR-31 and miR-155 preventing their damaging effect on the mucosa in UC.

Despite the fact that the main detrimental cytokine in UC is IL-13 produced by NKT cells, TNF-α expression has been shown to be up-regulated in UC and correlates to the grade of inflammation in untreated patients (Olsen, Goll et al. 2007). TNF-α is of vital importance in the pathogenesis of UC and CD and has been shown to induce both miR-31 and miR-155 expression (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007, Suarez, Wang et al. 2010).

mRNA expression of TNF-α was not significantly different in active UC samples as compared to normal biopsies or unaffected inactive UC, (***Figure 13***) which may well reflect the fact that most of patients have been on various treatments for UC. TNF-α is a direct target in IBD therapy with anti-TNF therapy such as Infliximab and Adalimumab. Such biologic therapies may therefore have a profound influence on the expression of these microRNAs in IBD tissues. The effect of drugs used in IBD therapy on the expression of microRNAs is yet unknown and will be discussed at a later point in this chapter (see ***11.2*** and ***11.3***).

As discussed before, IL13Rα1 has been reported to be up-regulated in epithelial cells isolated from UC resections (Mandal and Levine 2010), but our data shows a significant decrease of IL13Rα1 mRNA expression in active UC (endoscopic Mayo score 2-3) compared to paired unaffected inactive samples (***Figure 13***). This was confirmed with data from protein expression of IL13Rα1 in colonic biopsies which showed a significantly lower expression of IL13Rα1 in Western blots of active UC as compared to unaffected, inactive disease and normal controls (***Figures 14*** and ***15***). Isolates from epithelial cells of biopsies from normal controls, unaffected inactive UC and active UC also showed a significant decrease of IL13Rα1 mRNA in active samples as compared to normal and unaffected inactive UC epithelial cell isolates (***Figure 17***).

The increase of IL13Rα1 in UC (Mandal and Levine 2010) was not demonstrated in matched paired samples in biopsies such as our patient data with samples taken in the immediate proximity of inflamed and unaffected inactive tissue from the same patient, but in resection specimens which may explain the different results. The sample number in Mandal *et al*. study (Mandal and Levine 2010) was also much lower than in our study which makes our data of more statistical relevance and there was no information provided for the indication of colectomy or which medication patients have been on prior to resection. No grade of inflammation in UC samples was provided in this study.

In order to determine the direct targeting of the 3'UTR of IL13Rα1 by miR-31 and its possible synergistic action with miR-155 (which targets IL13Rα1 in a proximal site in the 3'UTR (Martinez-Nunez, Louafi et al. 2011)), a Dual-Luciferase Reporter Assay System (Promega) was set up (see ***section 2.12***) after cloning the 3'UTR of IL13Rα1 into the 3'UTR of a reporter gene (Renilla luciferase, pRLTK from Promega) and cloning miR-31 into pCDNA3.1 (see ***section 2.11***). This assay

demonstrated a significant 50% reduction of the Renilla Luciferase activity when co-transfected with pCDNA3.1-miR 31. Mutation of the predicted site abolished this effect proving the direct targeting of IL13Rα1 by miR-31 and mapping its binding site to position 1146 in the 3'UTR (***Figure 20***). Reduction of IL13Rα1 levels via direct targeting by miR-155 has been shown in published data from our group (Martinez-Nunez, Louafi et al. 2011).

The expression of several microRNAs (miR-27ab, miR-31, miR-152, miR-155, miR-183, miR-196ab, miR-324-5p and miR-374) predicted to target IL13Rα1 by three microRNA bioinformatics tools (TargetScan, Microrna.org, PITA algorithm, ***Figures 6*** and ***7***) was assayed in this study. This revealed differential expression in all of them comparing normal tissue, unaffected inactive UC and active UC in our patient population (***Figure 16***).

Apart from miR-27a and miR-374, all other microRNAs tested were previously reported to be deregulated in a study by Fasseu *et al* in patients with IBD (Fasseu, Treton et al. 2010). MiR-31 and miR-155, which were both highly significantly raised in our data in samples of patients with active UC as compared to unaffected inactive and normal tissue (***Figure 16***) and have been shown to be increased in UC in previous studies (Takagi, Naito et al. 2010, Olaru, Selaru et al. 2011).

The significance of the highly up-regulated expression of miR-31 and miR-155 is underlined by their important roles in the regulation of the innate and adaptive immune response and their importance in tumour biology.

As discussed before, MicroRNA-31 (miR-31), is located on chromosome 9p21.3 is in immediate proximity to a region on chromosome 9, where a cluster of interferons are situated (***Figure 4***). Interestingly, interferon-β-1a induces a clinical response and remission patients with UC that is associated with significant inhibition of IL-13 production facilitated via SOCS proteins (Mannon, Hornung et al. 2011). This suggests a possible regulation in common between miR-31 and the interferon cluster vicinity. MiR-31 has also been implicated to play a crucial role in inflammation preventing leucocyte recruitment and T-reg regulation, as well as exhibiting multiple functions in neoplastic disease. TNF-α, increased in our inflamed UC patient samples (***Figure 13***), induces miR-31 and has been shown to target E-selectin, which recruits neutrophils to be trans-located to a site of inflammation. Specific inhibition of miR-31 increased neutrophil adhesion to cultured endothelial cells, suggesting that miR-31 provides negative feedback control of inflammation

and has a potential role in anti-inflammatory therapy (Suarez, Wang et al. 2010), which could be applied to UC. MiR-31 is also directly involved in T-reg biology by directly targeting FOXP3 transcription factor, a master regulator of T-reg cell differentiation and function. This indicates that miR-31 antagonizes a T-reg phenotype by suppressing the expression levels of this important transcription factor (Rouas, Fayyad-Kazan et al. 2009).

In UC, miR-31 expression has been shown to change in a stepwise fashion as tissue goes from normal to chronically inflamed to actively inflamed to neoplastic and could have a role for stratifying patients with long term IBD with regards to cancer risk. MiR-31 expression was significantly increased in sporadic colorectal cancer specimens compared to normal specimens, although miR-31 levels were lower in sporadic colon cancers than in IBD-associated neoplasia (Olaru, Selaru et al. 2011). Several other studies have highlighted the increased presence of miR-31 in colorectal cancer (Slaby, Svoboda et al. 2007, 2009, Brereton, Bodger et al. 2010, Chang, Mestdagh et al. 2010, Earle, Luthra et al. 2010, Iacucci, de Silva et al. 2010, Velayos, Liu et al. 2010, Qualtrough, Smallwood et al. 2011, Ullman and Itzkowitz 2011). MiR-31 is also involved in the responsiveness of colorectal cancer cells to 5-FU, a chemotherapy agent for colorectal cancer. (Wang, Stratmann et al. 2010). MiR-31 has also a wide variety of further potential gene targets relevant to IBD such as FOXP3, CD28, histone deacetylase (HDAC) 1, HDAC2 and HDAC9 (Rouas, Fayyad-Kazan et al. 2009). This highlights the importance of this microRNA making it a potentially interesting player in IBD and opening options for future work of clinical relevance.

As previously discussed, miR-155 has been shown to have multiple functions within the innate and adaptive immune system. Similarly to miR-31, miR-155 is involved in tumour biology.
Our group found that miR-155 is a crucial regulator in macrophages through directly targeting IL13Rα1 and reducing levels of IL13Rα1 protein. This leads to diminished activation of STAT6 and affects the IL-13-dependent regulation of several genes (SOCS1, DC-SIGN, CCL18, CD23, and SERPINE1) involved in the establishment of an M2 phenotype in macrophages (Martinez-Nunez, Louafi et al., 2011). Published data from our group also established how miR-155 participates in the maturation of human dendritic cells (DC) and modulates their pathogen binding by down-regulating DC-SIGN through directly targeting the transcription factor PU 1. This suggests a mechanism by which miR-155 regulates proteins involved in the cellular immune response against pathogens (Martinez-Nunez, Louafi et al. 2009), essential in the gut immune balance.

Further published data from our group provided firm evidence of miR-155 directly repressing SMAD2 expression, demonstrating an important role for miR-155 in modulating the cellular response to TGF-β with possible implications in several human diseases where homeostasis of TGF-β might be altered such as asthma or IBD, and especially CD (Louafi, Martinez-Nunez et al. 2010).

As previously discussed, MiR-155 is significantly involved in the regulation of the innate immunity. It has been shown to regulate the acute inflammatory response after pathogen recognition by TLRs on monocytes or macrophages (Bakirtzi, Hatziapostolou et al. 2011). Expression of miR-155 was observed to be inducible during bacterial stimulation, as well as after exposure of cells to pro-inflammatory cytokines such as IFN-γ, IFN-β or TNF-α (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007). A positive correlation between miR-155 up-regulation and NF-κB activation, a transcriptional factor that regulates a host of genes that are critical to innate and adaptive immunity, cell proliferation, inflammation and tumour development, has been reported (Ma, Becker Buscaglia et al. 2011). This provides a theoretical link between inflammation and cancer in IBD patients There is convincing evidence to indicate that chronic inflammation is a key risk factor for CRC in patients with IBD (Farraye, Odze et al. 2010) .

MiR-155 maintains T-regs proliferation and homeostasis by the down-regulation of SOCS1 expression, in addition to directly targeting Foxp3 in T-regs and contributing to their development (Rodriguez, Vigorito et al. 2007, Kohlhaas, Garden et al. 2009). MiR-155 has also been shown to be involved in B-cell development and function (Chen, Li et al. 2004, Hu, Fong et al. 2010). Yao *et al*. demonstrated that miR-155 enhanced T-reg and Th17 cells differentiation and IL-17A production by targeting SOCS1 (Yao, Ma et al. 2012)

Together all this evidence links miR-155 to different inflammatory pathways and to the regulation of innate and adaptive immunity as well as tumour biology. MiR-155 could therefore be an important player in the pathogenesis of inflammatory bowel disease.

Both miR-155 and miR-31 seem to play potentially important roles in IBD, especially in UC, and in IBD associated colorectal cancer. These microRNAs have been shown to be up-regulated in our data in patients with active UC alongside a marked increase of IL-13 dependent genes and a

down-regulation of IL13Rα1. It is of particular interest for this study that both microRNAs target IL13Rα1, a crucial player in the pathogenesis of UC. *In vitro* models of colonic epithelial cells (HT-29 cells) and THP-1 cells (providing a model for lamina propria macrophages and dendritic cells) were used to investigate the role of miR-31 and miR-155 in the IL-13 pathway in UC, especially on the expression of IL13Rα1, SOCS1 and CCL26. We also aimed to determine whether there is a synergistic action between miR-31 and miR-155.

Using these two micro-RNAs (miR-31 and miR-155 and their combination) to target IL13Rα1 to reduce its expression was successfully demonstrated *in vitro* experiments in HT-29 colonic epithelial cells (***Figures 40, 41*** and ***42***), monocytic THP-1 cells (***Figure 49***) and human monocytes/macrophage cell models (***Figure 52***). The reduction of IL13Rα1 led to a significant reduction of expression of CCL18 (human monocytes, ***Figure 50***), SOCS1 (HT-29 cells, THP-1 cells, human monocytes, ***Figures 41, 47 and 51***, respectively) and CCL26 (HT-29 cells, ***Figure 42***) following stimulation with IL-13 in mRNA expression. We were also able to show a significant down-regulation of IL13Rα1 protein expression in epithelial colonic HT-29 cells by pre-miR-31 and pre-miR-155 and its combination which also led to a significant reduction in phosphorylation of STAT6 (***Figures 43*** and ***44***, respectively). Down-regulation of the JAK-STAT6 pathway with siRNAs has been shown to lead to a reduction of the toxic effects of IL-13 to epithelial cells (Rosen, Frey et al. 2011) underlining the biological relevance of our findings.

The combination of pre-miR-31 and pre-miR-155 at 50nM each in the mRNA expression of IL13Rα1 in all of our *in vitro* models did not suggest a particular synergy, but behaved similar to the full single dose of the individual microRNAs at 100nM (HT-29 cells, THP-1 cells and primary macrophages in ***Figures 40, 49*** and ***52***, respectively).

Therefore, based on our findings in paired unaffected inactive and active UC showing a significant down-regulation of IL13Rα1 on mRNA and protein level with corresponding significantly elevated levels of miR-31 and miR-155, and the ability to down-regulate the IL-13/STAT6 pathway with transfection of these microRNAs in our *in vitro* models, the transfection of biopsy samples in explant cultures from patients with active UC was the logic next step. The aim of explant culture transfections was to investigate if miR-31 and miR-155 may have therapeutic potential leading to reduction of pro-inflammatory effects of IL-13 pathway stimulation by reducing the IL13Rα1.

Transfection of active UC explant cultures (endoscopic Mayo score 2-3) with pre-miR-31 and pre-miR-155 and its combination clearly had an effect on IL13Rα1 significantly reducing its expression compared to a control sample at a dose of 100nM in the single transfection samples with pre-miR-31 and pre-miR-155, and a further reduction of the mRNA expression observed in the combination treatment group of pre-miR-31/155 at 50nM each (***Figure 53***). This effect also translated into the expression of the IL-13 dependent genes CCL18 and SOCS1 reducing their mRNA expression in an even more pronounced magnitude, especially using the combination of pre-miR-31 and pre-miR-155 (***Figures 54*** and ***55,*** respectively).

The reduction of IL13Rα1 by pre-miR-31 and pre-miR-155 and the combination of the two microRNAs leading to a mitigation of IL-13 dependant gene expression (CCL18, SOCS1) confirms our previous findings *in vitro* using cell models of the colonic epithelial cells (HT-29 cells), THP-1 cells and the human monocyte derived macrophages.

No significant changes were seen in the mRNA levels of SERPINE1 and CCL26, which can be explained by the presence of TGF-beta in the biopsy samples which also stimulates the expression of these genes apart from IL-13. We have shown the effects of TGF-beta stimulation on SERPINE1 and CCL26 in HT-29 cells (see ***Figure 22***). MMP9 was seen to be increased in all microRNA treatment groups and may just generally represent higher levels of MMP9 due to tissue degradation releasing increased levels of MMP9 (see ***Figure 55***). No significant differences were seen in the expression of TNF-alpha as compared to the control group and may indicate that the IL13 pathway does not significantly influence the expression of TNF alpha (***Figure 55***).

The finding of a potential anti-inflammatory effect of miR-31 and miR-155 through reduction of the IL13Rα1 expression leads to the conclusion that these microRNAs could have therapeutic use in ulcerative colitis.

A possible synergistic effect of miR-31 and miR-155 in active UC explant cultures is a novel finding not described before. Each individual microRNA separately and especially the synergistic combination of miR-31 and miR-155, influences the activation of IL-13 dependent genes and possibly the activation of the JAK-STAT6 pathway. The high levels of miR-31 and miR-155 in inflamed UC tissue causing a down-regulation of IL13Rα1 may well be an attempt to stop the activation of the IL-13/STAT6 pathway.

Overall taking the in vitro data from HT-29 cells and human monocytes/macrophages into account showing the potential to down-regulate IL13Rα1, as well as the data from the Explant Culture biopsy transfection provides evidence of the therapeutic potential of miR-31 and miR-155, each microRNA alone or in combination, in active human ulcerative colitis. The importance of IL-13 in the pathogenesis of ulcerative colitis is highlighted by the fact that anti-IL-13 drugs are currently being evaluated in clinical trials.

In summary, we have shown for the first time that miR-31 directly targets IL13Rα1, which is a known target of miR-155. Our results show up-regulated expression of IL-13 dependent genes in inflamed samples of patients with active UC in the presence of highly significantly increased expression of miR-31 and miR-155 as compared to normal and unaffected inactive patient samples. Despite the down-regulation of IL13Rα1 IL-13 dependent genes remain significantly up-regulated in active UC. IL13Rα1, SOCS1 and CCL18 expression can be down-regulated in *in vitro* models, but also in explant cultures of active UC biopsies by pre-miR-31 and pre-miR-155 and their combination. This could provide a therapeutic option in the treatment of UC in the future.

8.2 General discussion 2: Profiling and effects of therapy in microRNA and mRNA expression in IBD

8.2.1 UC profiling and effects of therapy in mRNA and microRNA expression

The series of UC sigmoid biopsies analysis was aimed to establish a differential expression profile of microRNAs (miR-31, miR-155 and miR-146a) involved in immunity and inflammation and mRNA (Th1, Th2 and tissue remodelling genes) between unaffected, inactive and active UC. Firstly, a profile was created not taking medication into account as a baseline. The data was then broken down into treatment naïve patients, patients who were taking 5-ASA medication and/or Thiopurines to highlight potential differential microRNA and mRNA expression influenced by these treatments for UC.

Our findings for microRNAs miR-31 and miR-155 in the pooled data (***Figure 56***) confirmed previous findings in paired UC samples of unaffected, inactive and active disease as shown in ***Figure 16*** and are in line with published literature on microRNA expression in UC (Fasseu, Treton et al. 2010, Takagi, Naito et al. 2010, Olaru, Selaru et al. 2011).

The significant increase of miR-146a in active UC as compared to unaffected, inactive samples is a novel finding not described in the literature before.

Th1 related genes (TNF-α, IL1β, IL2β and IL12βR1) did not play a major role in the pooled unaffected, inactive and active UC and apart from IL1β did not show and significant difference between unaffected, inactive and active sigmoid colon UC biopsies (***Figure 57***). The significant increase in inflamed UC samples of IL1β is in line with published literature (Brynskov, Tvede et al. 1992), but IL2β was not found to be sufficiently expressed in this series and showed no difference in expression. The pooled data did not show a previously reported significant increase of TNF-α in inflamed UC samples (Harvey, Naciti et al. 2004) as compared to unaffected, inactive biopsies which may be explained by the diversity of patient characteristics in relation to disease duration and medication, although no patients on anti-TNF-α treatment in UC were included in this study.

Th2 cells express only the IL-12Rβ1 chain (Presky, Yang et al. 1996) and findings in this series in unaffected, inactive and active UC sigmoid biopsies confirmed this. No significant expression of

IL-12Rβ2 in UC samples was seen with no difference between unaffected, inactive and active UC (***Figure 57***).

IFN-γ was not found to be expressed in unaffected, inactive or active UC which is in keeping with the distinctive lack of IFN-γ expression in UC (Strober and Fuss 2011).

Messenger RNA of genes involved in the Th2 response (CCL18, SOCS1, CCL26, IL13Rα1 and TSLP) revealed a significant up-regulation of CCL18, SOCS1 and CCL26 and a significant down-regulation of IL13Rα1 in active UC samples (***Figure 58***) as previously demonstrated in paired UC samples (***Figure 16***). Importantly, CCL18 has not previously been reported to be up-regulated in UC, but has been shown to be involved in the inflammatory response in various human diseases (Schutyser, Richmond et al. 2005)

SOCS1 has not previously been shown to be elevated in inflamed UC biopsies. In fact no difference was noted in a study from Japan (Miyanaka, Ueno et al. 2007), but the majority of patients in this study received steroids, which may have altered the expression of SOCS1 in this series.

CCL26 was significantly increased in the active UC group (***Figure 58***) which confirms previous work demonstrating elevated CCL26 levels in blood and colonic biopsies of patients with UC (Manousou, Kolios et al. 2010).

Data from the pooled unaffected, inactive and active UC series in this study showed a significant decrease in IL13R α1 in active UC biopsies (***Figure 58***). This is in keeping with high levels of miR-31 and miR-155 in the same samples in the study population (***Figure 56***). This project have shown that miR-31 directly targets the 3'UTR of IL13Rα1 (***Figure 20***) and our group has previously established that miR-155 induces translational inhibition of IL13Rα1 mRNA by directly targeting its 3'UTR (Martinez-Nunez, Louafi et al. 2011). Increased expression of IL13Rα1 and IL-13Rα2 were previously reported in patients with UC, who underwent colectomy (Mandal and Levine 2010). However, no grade of inflammation, no reason for colectomy or any other clinical information was given, such as whether patients received rescue therapy for severe colitis or underwent colectomy for dysplasia or cancer.

TSLP is an inflammatory Th2 regulator that is increased in the mucosal lesions of UC and increased TSLP expression by IECs may trigger exacerbation of UC (Tanaka, Saga et al. 2010). In

this series TSLP expression was not significantly affected by active inflammation in this series of sigmoid biopsies in UC (***Figure 58***).

Tissue remodelling and fibrosis related genes (SERPINE1, MMP9, MMP12, TGF-β1 and COL1A2) showed a significant up-regulation of SERPINE1 (***Figure 59***). High levels of SERPINE1 have been shown to prevent wound healing (Holubar and Harvey-Banchik 2007). MMP9 and MMP12 were significantly increased in active UC as compared to unaffected, inactive disease (***Figure 59***), which is in line with previous studies demonstrating an up-regulation of these MMPs in UC (Vaalamo, Karjalainen-Lindsberg et al. 1998, Garg, Vijay-Kumar et al. 2009).

TGF-β1 did not show a significant difference between unaffected, inactive and active UC sigmoid biopsies of the pooled data (***Figure 59***) despite having been reported to be elevated in active UC compared unaffected, inactive UC biopsies (Babyatsky, Rossiter et al. 1996). Interestingly, sub-analysis in the treatment naïve group showed a significance difference between unaffected, inactive and active samples confirming previous published data (Babyatsky, Rossiter et al. 1996).

No significant changes were observed in the pooled data of our UC patient group in the expression of COL1A2 in unaffected, inactive and active UC (***Figure 59***). COL1A2 previously has only been described to be up-regulated in a mouse model of colitis (Wu and Chakravarti 2007).

We believed that medication treatment had the potential to influence microRNA and mRNA expression and that pooling the samples could mask this effect. We therefore sought to analyse the expression of our candidate microRNAs (miR-31, miR-155 and miR-146a) and a panel of inflammatory genes as well as genes involved in tissue remodelling/fibrosis in the same patient samples, dividing them into sub groups according to their treatment and response.

Medication used to treat UC can influence the expression of microRNAs involved in immune responses and inflammatory pathways and also alters the mRNA expression of cytokines and mediators implicated in the Th1, Th2 and remodelling processes (***Figures 60-66***).

5-ASA is implicated in the down-regulation of the inflammatory-related Nuclear Factor κB (NF-κB) pathway (Barnes and Karin 1997) and the inhibition of T-cell proliferation (Fujiwara, Mitsui et al. 1990). Thiopurines exhibit anti-proliferative effects on activated lymphocytes, induce T-cell apoptosis and influence the NF-κB pathway (Tiede, Fritz et al. 2003, Schroll, Sarlette et al. 2005).

MiR-31, miR-155 and miR-146a have all been shown to play a significant role in T-cell biology and have been implicated in the regulation of the NF-κB pathway (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007, Bhaumik, Scott et al. 2008, Rouas, Fayyad-Kazan et al. 2009).

It is therefore very likely that expression profile of these microRNAs is altered by treatments that are targeting T-cells and the NF-κB pathway. Our results show that indeed medication treatment alters microRNAs 31, 155 and 146a levels.

The microRNA expression profile of miR-31, miR-155 and miR-146a was significantly influenced by treatment with 5-ASA and Thiopurines in patients with unaffected, inactive and active UC (***Figure 60***). 5-ASA and thiopurines generally increased the expression of miR-31, miR-146a and miR-155 in patients in remission on these drug treatments. The significant rise of these microRNAs in treatment naïve patients with active disease is lost in active UC samples treated with either 5-ASA or Thiopurines. MicroRNA levels of miR-31, miR-155 and miR-146a are generally lower or equal in active disease in both treatment groups as compared to treatment responders. MiR-31 and miR-155, both failing to increase significantly in both treatment groups in active UC, was reflected in the inability to decrease the IL13Rα1 significantly. This failure to up-regulate miR-31 and miR-155 could be an indicator of treatment resistance, as both have been shown to directly target IL13Rα1 (see ***Figure 20*** and (Martinez-Nunez, Louafi et al. 2011)). In explant cultures of active UC a significant down-regulation of IL13Rα1 was achieved by transfection of miR-31 and miR-155 and their combination (***Figure 53***) leading to an attenuation of genes activated in the IL-13 pathway (***Figure 54***).

Th1, Th2 and tissue remodelling genes were analysed in relation to the impact of 5-ASA and Thiopurines compared to treatment naïve patients in unaffected, inactive and active UC sigmoid biopsy samples (***Figures 61-66***).

The Th1 panel of genes tested consisted of TNF-α, IL1β and IL12Rβ1. TNF-α was significantly increased in unaffected, inactive and active treatment groups when compared to treatment naïve unaffected, inactive samples (***Figures 61*** and ***62***). The significant up-regulation in active samples of IL1β was lost in the treatment groups, but the expression was increased in both unaffected, inactive and active UC. MiR-146a has been implicated to be involved in the regulation of IL1β dependant pathways by directly targeting the IRAK1 (IL-1 receptor-associated kinase 1) (Chatzikyriakidou, Voulgari et al. 2010). It has been shown to negatively regulate the activation of IL1β dependant chemokines in lung epithelial cells (Perry, Moschos et al. 2008).

Mir-146a was increased by 5-ASA and Thiopurine treatment and associated with a loss of the significant difference of between unaffected, inactive and active samples (***Figure 60***). This might indicate that 5-ASA and Thiopurine treatment influence the IL1β pathway in UC.

TNF-α was not significantly different in treatment naïve unaffected, inactive and active UC sigmoid biopsies, but the expression was significantly raised in both treatment groups for 5-ASA and Thiopurines. No significant difference in the expression of TNF-α was observed between unaffected, inactive and active UC samples in the treatment groups (***Figures 61*** and ***62***). Previously reported increased expression of TNF-α in active UC (Harvey, Naciti et al. 2004) was not observed in this series.

No significant impact of treatment was seen in relation to IL12βR1 (***Figures 61*** and ***62***).

The Th2 panel of genes investigated in this series consisted of CCL18, SOCS1, CCL26, IL13Rα1 and TSLP (***Figures 63*** and ***64***).

CCL18 has been shown to be elevated in various inflammatory diseases (Schutyser, Richmond et al. 2005). CCL18, a chemokine not previously described to be elevated in IBD tissues, is significantly increased in active treatment naïve UC, but this response is lost in both 5-ASA and Thiopurine treatment groups. Interestingly, in both treatment groups CCL18 was significantly increased as compared to treatment naïve unaffected, inactive samples (***Figures 63*** and ***64***). Further research is needed to clarify the role of CCL18 in UC.

SOCS1, a direct target of miR-155 (Lu, Thai et al. 2009), was significantly up-regulated in active treatment naïve UC samples. This difference was lost in the treatment groups, but compared to treatment naïve unaffected, inactive samples SOCS1 levels were significantly higher (***Figures 63*** and ***64***). This loss of a significant rise in inflamed UC was in keeping with miR-155 levels in the medication groups (***Figure 60***). SOCS1 levels, on one hand seem to be associated with inhibition of STAT6 inflammation, but on the other hand also indicate continued activation of the IL-13 pathway. Higher levels of SOCS1 in 5-ASA and Thiopurine treated samples may indicate involvement in the up-regulation of SOCS1 of these medications. SOCS1, an inhibitor of STAT6 (Dickensheets, Venkataraman et al. 1999) has been associated with a clinical response and remission in a large subset of patients with UC by inhibition of IL-13 production after receiving Interferon-β-1a (Mannon, Hornung et al. 2011). The expression profile of SOCS1, reaching its maximum expression after 1 hour (Hebenstreit, Luft et al. 2003), seems to be an important

defence mechanism in UC, but may be overwhelmed by a strong IL-13 driven response typical for UC (Strober and Fuss 2011).

The significant down-regulation of the IL13Rα1 in treatment naïve active samples was lost in the treatment groups with 5-ASA and Thiopurines (***Figures 63*** and ***64***). This also corroborates with the loss of a significant difference of the directly targeting microRNAs miR-31 and miR-155 in the same samples in the treatment groups for both 5-ASA and Thiopurines (***Figure 60***). Interestingly, in patients who are in remission receiving Thiopurines, the IL13Rα1 levels remained at a low level comparative to the active treatment naïve group (***Figure 64***). This also corresponds with significantly elevated miR-31 levels, but not with miR-155 levels (***Figure 60***). Expression levels of the IL-13 pathway genes CCL18 and SOCS1 in the Thiopurine group (***Figure 64***), although significantly up-regulated compared to treatment naïve patients, were lower compared to non-responders in this group. In the 5-ASA responders the IL13Rα1 level was similar to the treatment naïve unaffected, inactive sample with a lesser effect on the expression of SOCS1 and CCL18. However, both SOCS1 and CCL18 were elevated as compared to treatment naïve samples (***Figure 64***). MiR-31 and miR-155 as well as 5-ASA and Thiopurines seemed to influence the expression of IL13Rα1 (***Figures 60, 63*** and ***65***). In explant cultures of active UC a significant down-regulation of IL13Rα1 was achieved by transfection of miR-31 and miR-155 and their combination leading to an attenuation of genes activated in the IL-13 pathway (***Figure 54***). This effect of a significantly down-regulated IL13Rα1 was observed in treatment naïve active samples and maintained in Thiopurine responders, but lost in non-responders (***Figure 64***). CCL26 behaved in a similar way to CCL18 and SOCS1, but did not reach significance (***Figures 63*** and ***64***). TSLP was not significantly different in treatment naïve unaffected, inactive and active samples and 5-ASA had no influence on its expression. Thiopurines in contrary, increased the expression significantly in treatment responders, and this increase was significantly down-regulated in non-responders (***Figure 64***). These findings are not in line with previously published data on increased TSLP expression in mucosal lesions of UC (Tanaka, Saga et al. 2010) and need further investigation.

The tissue remodelling and fibrosis panel of genes tested consisted of SERPINE1, MMP9, MMP12, TGF-β1 and COL1A2 (***Figures 65*** and ***66***). SERPINE1, MMP9 and MMP12 were all significantly increased in active treatment naïve sigmoid biopsies (***Figures 65*** and ***66***). This significant difference was lost in both treatment groups with 5-ASA and Thiopurines for SERPINE1 and MMP9, but in non-responders the mRNA levels of all 3 genes remained significantly elevated (***Figures 65*** and ***66***). High levels of SERPINE1 have been shown to be

associated with impaired wound healing (Holubar and Harvey-Banchik 2007). Significant up-regulation in treatment naïve active and more so in treatment non-responders to both 5-ASA and Thiopurine treatment (***Figures 65*** and ***66***) might be prognostic markers for treatment resistant active UC. Treatment responders in both groups show SERPINE1 levels similar to unaffected, inactive treatment naïve samples (***Figures 65*** and ***66***).

TGF-β1 was significantly up-regulated in active treatment naïve UC biopsy samples as compared to treatment naïve unaffected, inactive samples (***Figures 65*** and ***66***). This is in line with published literature showing that TGF-β1 is elevated in active UC compared unaffected, inactive UC biopsies (Babyatsky, Rossiter et al. 1996). Treatment with 5-ASA abolished this difference between unaffected, inactive and active tissue. In the case of Thiopurines, this effect seen in treatment naïve patients was reversed with a significant down-regulation of TGF-β1 in non-responders not only compared to unaffected, inactive treated samples, but also compared to active treatment naïve samples. TGF-β1 was also significantly up-regulated in treatment responders in the Thiopurine group (***Figure 66***) as compared to treatment naïve patients which might indicate an important role for TGF-β1 in achieving remission in UC.

COL1A2 showed no difference in expression in treatment naïve patients, but was elevated in both treatment groups (***Figures 65*** and ***66***), reaching significance in the Thiopurine group in non-responders (***Figure 66***).

In summary, 5-Aminosalicylates and Thiopurines altered the expression of microRNAs (miR-31, miR-146a and miR-155) and mRNA expression of Th1, Th2 and tissue remodelling and fibrosis genes substantially. This needs to be taken into account conducting research in tissue biopsies in UC. Furthermore, multi-variant analysis of this data in UC could identify a profile of microRNAs and mRNA which could be potentially be used to predict response or failure to treatment with these drugs.

8.2.2 Crohn's profiling and effects of therapy in mRNA and microRNA expression

Our series of CD sigmoid biopsies, similar to previous data in UC in this chapter, was intended to ascertain a differential expression profile of microRNAs (miR-31, miR-155 and miR-146a) involved in immunity and inflammation and mRNA (Th1, Th2 and tissue remodelling genes) between unaffected, inactive and active CD. Firstly, a profile was created not taking medication into account as a baseline. The data was then broken down into treatment naïve patients or patients who were taking Thiopurines and/or anti-TNF-α treatments. The aim was to highlight potential differential microRNA and mRNA expression influenced by these treatments in patients with unaffected, inactive and active sigmoid CD.

Our findings in the overall pooled data showed a significant up-regulation in the expression of miR-31, miR-146a and miR-155 in active disease compared to unaffected, inactive sigmoid biopsies (***Figure 67***), even more pronounced as observed in UC (***Figure 56***) in this thesis. Increased expression of miR-31 miR-146a and miR-155 has been previously reported in unaffected, inactive and active CD (Fasseu, Treton et al. 2010, Takagi, Naito et al. 2010, Olaru, Selaru et al. 2011)

Th1 related genes TNF-α, IL1β, IL12βR1, IL12βR2 and γ-IFN were assayed and their expression was found to be in line with previously published data (***Figure 68***). Raised TNF-α in inflamed mucosa of CD patients was reported to be a cornerstone in the pathogenesis of CD (Reimund, Wittersheim et al. 1996). IL1β has been shown to be increased in active CD (Brynskov, Tvede et al. 1992) and IL12βR2 has also been shown to be up-regulated in active CD (Parrello, Monteleone et al. 2000). Interestingly, IL12Rβ1, which was also significantly up-regulated in this series in active CD, is a predicted target of miR-146a by TargetScan (www.targetscan.org). This may be suggestive of a role for miR-146a in the IL-12 pathway in Crohn's disease. Increased miR-146a in active CD did not seem to be able to down-regulate IL12Rβ1 in this series. High IFN-γ expression was previously reported in active CD (Kugathasan, Saubermann et al. 2007).

Pooled Th2 genes (CCL18, SOCS1, CCL26, IL13Rα1 and TSLP) assayed showed a significant up-regulation in active sigmoid CD of CCL18, SOCS1 and CCL26, whereas IL13Rα1 was significantly down-regulated, similar to the findings in UC. TSLP was not significantly different in unaffected, inactive and active CD (***Figure 69***). CCL18 has not previously been described in CD and findings showing an up-regulation in active CD are novel. SOCS1 has been shown to be differentially

expressed in CD, but the study was conducted on paraffin fixated specimens (Christophi, Rong et al. 2012). CCL26 has been shown to be moderately up-regulated in active CD, but less compared to active UC (Takahashi, Imaeda et al. 2013). IL13Rα1 expression in CD is not well described, but its significant down-regulation in active CD (***Figure 69***), may correlate with the up regulation of miR-31 and mir-155 (***Figure 67***), which directly target IL13RA1 (***Figure 20*** and (Martinez-Nunez, Louafi et al. 2011). Recently increased expression of IL13Rα1 in transitional cells lining CD fistulas was reported and IL-13 was implicated to play a major role in fistula formation (Scharl, Frei et al. 2013). TSLP, a regulator of the Th2 response, was not significantly differentially expressed in unaffected, inactive and active CD mucosa from the sigmoid.

Fibrosis and tissue remodelling genes (SERPINE1, MMP9, MMP12, COL1A2 and TGF-β1) were all significantly increased in active CD as compared to unaffected, inactive CD sigmoid mucosal biopsies (***Figure 70***). SERPINE1 has been reported to be increased in CD as compared to normal controls (Miseljic, Galandiuk et al. 1995). MMP9 and MMP12 have been shown to be up-regulated in IBD (Pender, Li et al. 2006, Garg, Vijay-Kumar et al. 2009) and our findings in CD corroborate with previously published research in the pooled CD data. COL1A2, a gene encoding for type 1 collagen, has not previously been published to be elevated in CD, but its increase in active CD samples in this series may indicate a pro-fibrotic role in inflammation. TGF-β1 has been well described to be elevated in CD and confirms the findings in this study. TGF-β1 was shown to be elevated in both active CD compared to normal controls and unaffected, inactive CD samples (Babyatsky, Rossiter et al. 1996).

To answer our question about a possible effect of drug therapy in microRNA and mRNA expression in CD, we divided our samples taking into account the medication and response to treatment after analysing the microRNA and mRNA expression in the pooled samples.

Our results show that medication used to treat CD can manipulate the expression of microRNAs involved in immune responses and inflammatory pathways and also influences the mRNA expression of cytokines and mediators implicated in the Th1, Th2 and remodelling processes (***Figures 71-77***).

Thiopurines exhibit anti-proliferative effects on activated lymphocytes, induce T-cell apoptosis and influence the NF-κB pathway (Tiede, Fritz et al. 2003, Schroll, Sarlette et al. 2005). Anti-TNF-α compounds (Infliximab and Adilumimab) are anti-TNF-α monoclonal antibodies that have been shown to decreased expression of TNF-α, IL-1β and INF-γ in mucosal biopsies in patients

with CD (Ljung, Axelsson et al. 2007). The impact of these treatments on the expression of microRNAs in IBD has not yet been reported in the literature.

MiR-31, miR-155 and miR-146a, shown to be elevated in CD (Fasseu, Treton et al. 2010), have all been demonstrated to play a major role in T-cell biology and have been implicated in the regulation of the NF-κB pathway and other inflammatory pathways (Taganov, Boldin et al. 2006, O'Connell, Taganov et al. 2007, Bhaumik, Scott et al. 2008, Rouas, Fayyad-Kazan et al. 2009).

A significant up-regulation of all 3 microRNAs tested was observed in active samples of treatment naïve patients (***Figure 71***), but this effect was reversed by Thiopurine treatment in the case of miR-31 and miR-155, where significantly higher expression was seen in treatment responders as compared to treatment naïve active samples. MiR-146a was significantly reduced, compared to active treatment naïve disease, but still expressed at a higher level compared to treatment naïve patients with unaffected, inactive disease. Interestingly, in treatment non-responders, levels of all 3 microRNAs were comparable to unaffected, inactive treatment naïve patients, even significantly lower in the case of miR-31. The overall picture of the effect of Thiopurines on miR-31, miR-146a and miR-155 is not dissimilar to the profile observed in UC (***Figure 60***).

This is in complete contrast to the profile observed in patients treated with anti-TNF-α compounds, where in responders expression levels of all three microRNAs assayed returned to the levels seen in treatment naïve unaffected, inactive patients, or where even significantly lower as in the case of miR-146a. In contrast, non-responders showed significantly elevated levels of miR-31 and miR-155 compared to all other groups and only miR-146a was expressed less than in active treatment naïve patients, but its level was still significantly raised compared to treatment naïve unaffected, inactive patients (***Figure 60***).

This very diverse profile of microRNAs in the two treatment groups with Thiopurines and anti-TNF-α treatments is surprising, but highlights the complexity of the interaction between microRNAs, medication and the immune system. We suggest that persistently high levels of miR-31, miR-146a and miR-155 could serve as a marker for therapy resistance to anti-TNF-α treatment.

Th1, Th2 and tissue remodelling genes were analysed in relation to the impact of Thiopurines and anti-TNF-α treatment compared to treatment naïve patients in unaffected, inactive and active CD sigmoid biopsy samples (***Figure 72-77***).

The Th1 panel of genes tested consisted of TNF-α, IL1β, IL12βR1, IL12βR2 and INF-γ. TNF-α, IL1β, IL12βR1 and INF-γ were all significantly up-regulated in active treatment naïve samples as reported in the literature (Brynskov, Tvede et al. 1992, Reimund, Wittersheim et al. 1996, Parrello, Monteleone et al. 2000, Kugathasan, Saubermann et al. 2007), (***Figures 72*** and ***73***).

Both Thiopurines and anti-TNF-α treatments were able to significantly down-regulate TNF-α, IL1β, IL12βR1 and INF-γ in treatment responders, but higher levels of these genes in the active treatment group indicated treatment failure (***Figures 72*** and ***73***). IL12βR2 interestingly was only slightly more expressed in active treatment naïve CD, but not significantly increased compared to unaffected, inactive treatment naïve samples. IL12βR2 has been described as the crucial receptor in regulating Th1 differentiation in CD and was shown to be elevated in inflamed CD (Parrello, Monteleone et al. 2000). The highest levels of IL12βR2 were seen in non-responders to treatment in the Thiopurine and anti-TNF-α group, where it was significantly elevated in keeping with the literature (***Figures 72*** and ***73***). Ljung et al reported a decreased immune-histochemical expression of TNF-α, IL-1β and INF-γ in mucosal biopsies in patients with Crohn's disease after treatment with infliximab (Ljung, Axelsson et al. 2007), which is in line with the findings in this study for treatment responders to anti-TNF-α treatment. Failure to control expression of Th1 related genes by medication seems to an indicator of treatment resistance.

As previously mentioned, miR-146a could potentially play a role in regulating the IL-12 pathway as it is predicted to target IL12βR1 (www.targetscan.org). MiR-146a has been implicated to be involved in the regulation of IL1β dependant pathways by directly targeting the IRAK1 (IL-1 receptor-associated kinase 1) (Chatzikyriakidou, Voulgari et al. 2010). It has been shown to negatively regulate the activation of IL1β dependant chemokines in lung epithelial cells (Perry, Moschos et al. 2008) and may also play a role in the regulation of IL1β in CD.

The panel of Th2 related genes in CD consisted of CCL18, SOCS1, CCL26, IL13Rα1 and TSLP and were assayed to investigate the effects of Thiopurines and anti-TNF-α treatments on their expression in unaffected, inactive and active CD sigmoid biopsies. In treatment naïve patients CCL18, SOCS1, CCL26 and TSLP were significantly up-regulated and IL13Rα1was significantly down-regulated. The low levels of IL13Rα1 in active treatment naïve patients were associated with high levels of the directly targeting microRNAs miR-31 and miR-155 (***Figure 71***), similar to the expression profile in UC (***Figure 60***).

Differences in the expression of CCL18, SOCS1, CCL26, IL13Rα1 and TSLP between unaffected, inactive and active samples were diminished in patients treated with Thiopurines. Thiopurines were able to up-regulate IL13Rα in treatment responders and significantly down-regulate CCL18 and TSLP. Anti-TNF-α agents showed a stronger effect on Th2 genes and were able to down-regulate CCL18, SOCS1, CCL26 and TSLP significantly, while significantly up-regulating IL13Rα1. Recent reports of the importance of IL-13 in the formation of fistulae in CD (Scharl, Frei et al. 2013), together with the general rise of Th2 related genes in active CD (***Figure 69***) might suggest a greater role for Th2 related pathway genes than previously anticipated. This may contribute to the pathogenesis of CD and the ability of Thiopurines and anti-TNF-α treatments to significantly modulate Th2 related genes highlights their potential importance.

The panel of genes associated with fibrosis and tissue remodelling comprised of SERPINE1, MMP9, MMP12, TGF-β1 and COL1A2. The imbalance between inflammation and healing in CD is highlighted by complications of the disease with the formation of ulcers and fistulas on one hand and fibrosis and strictures on the other (Rieder, Brenmoehl et al. 2007). The complex nature of tissue remodelling in CD and the effect of medications used to treat CD is discussed in this section.

In treatment naïve patients a significant rise of SERPINE1, TGF-β1 and COL1A2 was observed in active sigmoid CD samples compared to unaffected, inactive mucosa (***Figures 74*** and ***75)***. MMP9 and MMP12 were interestingly not significant different, but there was a trend of up-regulation in active CD samples in treatment naïve patients. SERPINE1, MMP9, MMP12 and TGF-β1 have all been shown to be up-regulated in CD (Miseljic, Galandiuk et al. 1995), (Babyatsky, Rossiter et al. 1996).

Thiopurines had a profound impact on the expression of SERPINE1 down-regulating it significantly in treatment responders (***Figure 74***). High levels of SERPINE1 have been shown to impair wound healing and also promote fibrosis (Holubar and Harvey-Banchik 2007). COL1A2 and TGF-β expression in treatment responders is also significantly lower as compared to treatment naïve patients, which implies an anti-fibrotic effect which could be beneficial to prevent stricture formation in CD. MMP9 and MMP12 are less regulated by Thiopurines, but treatment non-responders have higher levels compared to all other groups, indicating on-going extra cellular matrix degradation (***Figure 76)***.

Anti-TNF-α treatments were able to significantly decrease the activity of SERPINE1, MMP9, MMP12, TGF-β1 and COL1A2 in treatment responders compared to active treatment naïve patients (***Figures 75*** and ***77***) indicating a major modulating role in preventing fibrosis and attenuation of tissue remodelling in CD. Interestingly pro-fibrotic genes such as SERPINE1, COL1A2 and TGF-β remain at relatively low levels in non-responders, while MMP9 and MMP12 up-regulation in non-responders may indicate persistent extra cellular matrix degradation. Previously, down-regulation of MMPs was demonstrated in CD biopsies of patients treated with Infliximab, with absence of this effect in non-responders (Di Sabatino, Saarialho-Kere et al. 2009).

Overall, similar to UC, microRNAs involved in inflammatory pathways and T-cell biology and mRNA expression of genes involved in Th1, Th2 and fibrosis/tissue remodelling pathways are significantly modulated by Thiopurines and anti-TNF-α treatment. In patients not responding to treatment generally these effects are significantly less pronounced and further analysis taking the microRNA and mRNA expression profile of these patients into account could potentially predict treatment failure and lead to change in therapy.

9 Conclusions

In summary, this study shows for the first time that miR-31 directly targets IL13Rα1, which is a known target of miR-155. Data presented demonstrates an up-regulated expression of IL-13 dependent genes in whole inflamed sigmoid biopsies and epithelial cell extracts from patients with active UC. Simultaneously there is a highly significantly increased expression of miR-31 and miR-155 as compared to normal and unaffected inactive patient samples. Despite significant down-regulation of IL13Rα1 (protein and mRNA expression) by direct targeting of miR-31 and miR-155, IL-13 dependent genes remain significantly up-regulated in active UC. IL13Rα1, CCL18, SOCS1 and CCL26 expression can be significantly down-regulated in three *in vitro* models comprising colonic epithelial cells, THP-1 cells and monocyte derived human macrophages, by transfection with pre-miR-31, pre-miR-155 and their combination. Protein expression of IL13Rα1 and IL-13-dependent phosphorylation of STAT6 was also successfully decreased *in vitro* by transfecting colonic epithelial cells with pre-miR-31 and pre-miR-155. Additionally, it was possible to make a connection back to colonic biopsy samples from patients with active colonic UC: IL13Rα1 can be successfully down-regulated in explant cultures to mitigate IL-13 dependent gene expression (CCL18 and SOCS1) by transfecting pre-miR-31 and pre-miR-155. This opens the possibility of a future therapeutic option in the treatment of UC.

MicroRNA expression of miR-31, miR-146a and miR-155 and mRNA expression of genes implicated in Th1 (TNF-α, IL1β, IL12βR1, IL12βR2 and INF-γ), Th2 (CCL18, SOCS1, CCL26, IL13Rα1 and TSLP) and in fibrosis/tissue remodelling (SERPINE1, MMP9, MMP12, TGF-β1 and COL1A2) related pathways are modulated by drugs used to treat UC and CD. Medication such as 5-ASA in UC, Thiopurines in UC and CD and anti-TNF-α compounds in CD can successfully modulate these microRNAs and mRNAs in treatment responders. Non-responders to treatment often fail to alter the microRNA and mRNA profile associated with active disease. Considering that microRNA and mRNA expression is modulated by treatment, it is important to underline that the results obtained in ***Chapter 4*** could have been more pronounced. In treatment naïve patients the results for microRNA and mRNA expression are more prominent so it is tempting to hypothesize that it is necessary to take into account medical treatment when performing experiments and analysis of IBD samples. Knowing the effects of these drugs on the microRNA and mRNA profile in unaffected inactive and active disease as well as evaluating the different expression profile

compared to treatment naïve patients might lead to a better understanding of treatment success or failure. Increasing the size of the study population and applying advanced statistical tools may aid to identify patients who are more like to respond or fail a treatment. Furthermore, monitoring of some of these microRNAs and mRNAs may lead to the identification of biomarkers which could contribute to improve and facilitate patient treatment.

10 Future work

10.1 Potential of miR-31 and miR-155 as UC therapy

Down-regulation of the IL13Rα1 with directly targeting microRNAs miR-31 and miR-155 could provide an adjunct to conventional treatment in patients with UC. Emerging treatments such as anti-IL-13 agents are currently evaluated in clinical trials highlighting the importance of the IL-13 pathway in the pathogenesis of UC. Furthermore, extensive studies using these microRNAs in Explant Culture models and potentially in animal models may be able to further define their role. MiR-31 and miR-155 could potentially be delivered as local therapy to work directly on the mucosa or could be packaged into acid resistant slow release medication form to be effective in the colon of patients with active UC.

10.2 Extensive profiling of IBD and effect of treatment in IBD

Establishing a profile of the effects of IBD therapy on pathways deregulated in IBD and the expression of microRNAs involved in vital processes of inflammatory pathways and T-cell pathology could provide new insight into treatment choice and may be able to predict treatment failure. Comparing the impact of these treatments and new emerging IBD therapies in patients with IBD prior and after starting treatment may be a way of homogenising the IBD cohort and would give more accurate information about time points within the IBD patient journey and this could be undertaken in a prospective manner. This could provide data regarding treatment response and may lead to identification of markers associated with response and non-response to treatment.

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