The role of microRNAs in Inflammatory Bowel Disease

Doctor of Philosophy Thesis

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UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

DEPARTMENT OF CLIINICAL AND EXPERIMAENTAL SCIENCES

Doctor of Philosophy

THE ROLE OF MICRORNAS IN INFLAMMATORY BOWEL DISEASE

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Inflammatory bowel disease has complex and as yet not fully understood immune pathways. It is thought that inflammatory bowel disease develops in genetically predisposed individuals, with dysregulated immune responses to unknown environmental triggers. Manipulation of the immune pathways by biological monoclonal antibodies has not proved to be the panacea that it had been hoped for.

MicroRNAs are single stranded RNA molecules which influence the translation of messenger RNA and hence protein synthesis. The altered expression of microRNAs in disease states in cancer and autoimmune diseases including inflammatory bowel disease is providing new insights into disease pathogenesis. This understanding has led to consideration of the utility of microRNAs in diagnostics, prognostics, and therapeutics in inflammatory bowel disease.

As yet, research involving analysis of microRNA profiles in inflammatory bowel disease, has focussed on heterogeneous groups of patients. Studies have not taken in to account the therapy or disease severity of the subjects studied. Also, although there has been research looking at the dysregulated expression of microRNAs, the role individual microRNAs are playing in the disease and how they could be used therapeutically has not been explored.

Using mucosal biopsies from patients on specific therapies, I performed RNA extraction and real time qPCR performed to analyse the profiles of microRNAs 31, 146a, and 155, and the target genes TNFα, SOCS1, and CCL18, in robustly phenotyped patients on conventional and biological therapies for ulcerative colitis, Crohn's colitis, and small bowel Crohn's disease. I then designed an ex vivo model to assess the effects of these therapies on microRNA and target gene expression, in treatment naïve tissue.

I go on to investigate the specific interaction of microRNA 31, and Thymic Stromal Lymphopoetin (TSLP) in the dysregulated IL-13 pathway in ulcerative colitis. I do this by performing both microRNA 31 and TSLP mRNA expression by qPCR in samples from a clinical trial using anti-IL13 monoclonal antibody, and by measuring TLSP protein concentrations in mucosal biopsies by Enzyme Linked Immunosorbant Assay. I then investigate the direct targeting of TSLP by microRNA 31 by Dual Luciferase analysis.

I show in this research that conventional and biological therapies have an important influence on the expression profiles of microRNAs and the target genes, and need to be taken into account when investigating microRNAs in inflammatory bowel disease. I also show that my ex vivo model can replicate the expression profiles seen in the treatment of inflammatory bowel disease, and could be utilised as a model for individualised medicine in the future. With this in mind, microRNAs are important in the immunoregulation of the bowel epithelium and are dysregulated in inflammatory bowel disease. MicroRNAs could possibly be utilised as diagnostic markers, predictive markers for response to treatment, or as future targets for novel therapies.

I go on to show that increased microRNA 31 could predict response to a novel anti-IL13 monoclonal antibody, and hypothesise that this therapy could better be used as maintenance of remission therapy rather than as an induction agent. I also show that microRNA 31 is associated with decreased expression of TSLP mRNA and protein, in active moderate to severe ulcerative colitis. I show that by Dual Luciferase analysis microRNA 31 directly targets and inhibits TLSP. The microRNA 31/TSLP immune regulation in ulcerative colitis could be a target for future therapies.

Academic Thesis: Declaration Of Authorship

I, Simon Whiteoak, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

The Role of microRNAs in Inflammatory Bowel Disease

I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University;
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
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Abbreviations

3'UTR: 3' UnTranslated Region

5-ASA: 5-AminoSalicylic Acid

AZA: azathioprine

CCL18: Chemokine (C-C motif) Ligand 18

CD: Crohn's Disease

CDAI: Crohn's Disease Activity Index

cDNA: Copy DNA

DGR8: DiGeorge Syndrome Critical Region 8

ELISA: Enzyme linked immunosorbant assay

GWAS: Genome-Wide Association Studies

HBV: Hepatitis B Virus

HCV: Hepatitis C Virus

HCC: Hepatocellular Carcinoma

HLA: Human Leukocyte Antigen

IBD: Inflammatory Bowel Disease

IFN-γ: Interferon gamma

IGA, IgA: Immunoglobulin A

IL-n: Interleukin - "number"

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

IL13Rα2: Interleukin 13 Receptor alpha 2 (mRNA or gene)

MIP 2α: Macrophage inflammatory peptide 2α

miR: microRNA

mRNA: messenger RNA

NF-κB: Nuclear Factor Kappa B

NKT: Natural Killer T cells

NOD2: Nucleotide-binding Oligomerization Domain containing 2

PAMPs: Pathogen-associated molecular patterns

PSC: Primary Sclerosing Cholangitis

RISC: RNA-Induced Silencing Complex

RT-qPCR: Reverse Transcription followed by Quantitative PCR

SMAD3: SMAD family member 3

SNP: Single Nucleotide Polymorphism

SOCS1: Suppressor of Cytokine Signalling 1

STAT3: Signal Transducer and Activator of Transcription 3

Th1: T Helper 1

Th2: T Helper 2

TSLP: Thymic Stromal Lymphopoeitin

TNF-α: Tumour Necrosis Factor alpha

T-regs: T regulatory cells

UC: Ulcerative Colitis

1. Introduction

1.1 Inflammatory Bowel Disease

1.1.1 Introduction

Inflammatory Bowel Disease (IBD) is a chronic inflammatory condition that is classically marked by periods of remission and relapse. IBD exists in two major forms, Crohn's disease (CD) and ulcerative colitis (UC). CD can affect any part of the gastrointestinal tract, whereas UC is confined to the large intestine. There is overlap between the two diseases with both diseases having commonality in the pathogenesis, being derived from a dysregulated immune response to microbiota within the gastrointestinal tract in genetically susceptible individuals. This dysregulation in immune response is the target of the new therapies in IBD.

IBD is more common in the West and in Europe has a higher incidence in Northern Europe although this gap is reducing(*1*). The incidence of CD is rising whereas the incidence of UC appears to have plateaued(*2*). The UK prevalence of inflammatory bowel disease is about 400 per 100000, with UC the more common disease with approximately 2/3 of the total IBD prevalence(*3*). Approximately 2.5-3 million people in Europe are affected by IBD. This has a direct healthcare cost of approximately £4.0-4.8 billion/year across Europe(*4*).

Cortisone was the first effective treatment for inflammatory bowel disease and was first used in the 1950s(*5*). Since then there have been many more treatments for inflammatory bowel disease with variable efficacy and side effect profiles. The monoclonal antibodies including the anti-TNF therapies have shown to be effective treatments in some patients but there is still a large therapeutic gap. Therefore, a lot of current research is into understanding the immunology of IBD and thus create more effective treatment strategies.

1.1.2 Pathogenesis of Inflammatory bowel disease

1.1.2.1 Genetic Basis

It has been known for over 50 years that there is a strong genetic factor in the pathogenesis of IBD. 10-30% of patients will have a family history of IBD. CD has a stronger familial concordance than UC; twin studies have demonstrated this with 50% in monozygotic twins for CD and 10-15% for UC. Genetic factors also influence disease phenotype(*6*).

Both CD and UC are complex polygenic diseases that do not follow simple Mendelian inheritance, with this in mind no one gene can be said to be a causative mutation for the development of IBD. The genome wide association study has identified multiple loci on multiple chromosomes that are involved in the development of IBD. The mechanism by which these genetic factors influence the development of IBD is not clearly understood.

The HLA status of patients does confer some risk, for example HLA-B27 is increased in a group of patients with UC and ankylosing spondylitis, but this is by no means absolute. Other studies have shown that HLA state can confer risk with the development or disease course and phenotype of UC and CD(*7-10*).

The first of these loci to be discovered was NOD2 gene located on chromosome 16 in 2001(*11, 12*) (also known as CARD15 and IBD1 gene loci). These mutations are seen in up to 40% of patients with CD and these patients present at an earlier age, and tend to have a more aggressive disease path requiring higher rates of surgery(*13*), specifically due to stricturing disease. NOD2 is a Pattern Recognition Receptor. It acts by sensor recognition of intestinal bacterial wall products that invade the epithelial barrier and triggers mucosal defence mechanisms. Its exact mechanism is unclear. NOD2 is thought to induce autophagy in dendritic cells by modulating antigen presentation and bacterial processing(*14*).

Since this discovery more than 150 loci associated with IBD have been identified(*15, 16*). The Genome Wide Association studies (GWAS) has been very influential in the understanding of the genetics of IBD, showing a picture of the genetic variants seen in UC and CD specifically and those gene defects seen across IBD as a whole, as shown in table 1.

UC	IBD	CD
ECM1 IL10 IFNγ IL22	IL23R IL12/p40 JAK2 STAT3	NOD2 ATG16L1 C11orf30
FCGR2A IL2/IL21 IL17/PIM3	NKX2.3 3p21/MST1	ZNF365 CCR6 1q24 6q21 7
CAPN10/GPR35	REL/PUS10 ZMIZ1 MTMR3	p12 13q14 ICOSLG GPX4
LAMB1/SCL26A3	IRGM ORMDL3 21q21 MHC	CCL2/CCL7/CCL11/CCL8 GCKR
OTUD3/PLA2G2E ARPC2	IL18RAP LYRM4 CDKAL1	SLC22A23 NLRP3 PTPN22
HN4α CDH1 IL26 13q12	TNFRSF6B PSMG1 CCNY IL27	LRRK2 1q32 6q25 8q24
1p36 CARD9 CEP72/TPPP	IBD5 PUS10 TNFSF15 19q13	18q11 ITLN1 ORMDL3
13q13 SMURF1/KPNA7	HORMAD2/MTMR3 PTGER4	SMAD3 ERAP2

Table 1. Overview of IBD susceptibility genes identified. Adapted from Thompson and Lees 2011(17)

1.1.2.2 Immunological Basis

It is likely that the genetic factors that influence the development of IBD have functional effects on the innate and adaptive immune systems and the intestinal epithelial barrier, as well as influencing the composition of intestinal microbiota.

The innate immune system is a rapid response pattern recognition system responding to antigenic components. Classically the structures that are used by cells in an innate immune response are the toll-like receptors. These respond to pathogen-associated molecular patterns (PAMPs) such as microbial DNA or lipopolysaccharides. These innate pattern recognition receptors are expressed on nearly all cell types, but especially dendritic cells, macrophages , B cells, and antigen presenting cells (including intestinal epithelial cells)(*18*).

The adaptive immune system is the "memory" of the immune system and therefore has a delayed response. This is characterised by T-cell response following the uptake, and presentation of antigens by antigen presenting cell to them.

The innate and adaptive immune systems work together regulating each other to promote a balanced immune response.

CD is characterised by a predominantly Th1 response, and release of their respective cytokines. Thus, increased IL-12, IL-17, IL-23, interferon-γ, and TNF-α are present. The presence of granulomas is a well-known consequence of Th1 response. The response of biological therapies against these cytokines is evidence for this response(*19-21*). Th1 activated cells interact with the antigen presenting cell to produce secretion high levels of TNF-α. This interaction makes TNF-α a very important cytokine and the most common target in the treatment of CD, most commonly Infliximab and Adalimumab.

UC affects only the superficial mucosa of the colon in contrast to Crohn's disease which is characterised by transmural inflammation, and the immunologically response is different to that of CD. It is characterised by cytokines secreted during a Th2 type response. Thus, here increased levels of IL-5 and IL-13 are seen. Although cytokines secreted by antigen presenting cells are also seen here as in CD, such as TNF-α, and IL-6. The increased levels of IL-13 primarily result from Natural Killer Cells(*22*). lL-13 is highly inflammatory and is mainly thought to be associated with the induction of airways disease and allergy. Along with TNF-

α and IL-6, IL-13 could be a cytokine that is responsible for the dysregulation of the epithelial barrier and tight junctions that leads to the increased permeability that could introduce antigens to the lamina propia causing inflammation(*23*). This pathway is dependent on many other genes such as chemokine ligands 18 and 26 (CCL18) (CCL26) and suppressor of cytokine signalling 1 (SOCS1).

IL-13 has already been shown to be upregulated in UC, and it induces the JAK-STAT6 via its receptor IL13Rα2(*24*), to which it has high affinity. SOCS1 is an inhibitor of the JAK-STAT6 induction along with Th1 interferons and down regulates IL-13 regulation(*25*). A study has shown that upregulation of SOCS1 and Th1 interferons, and therefore downregulation of IL13, may induce remission and clinical response to patients with UC(*26*).

Although therapies targeting TNF-α have been shown to be effective in UC, part of this research will study the effects of targeting IL-13 in an ex-vivo model for both UC and CD.

Thymic stromal lymphopoietin (TSLP) is another cytokine secreted by epithelial cells specifically associated to Th2 type response and is enhanced in allergic airways disease and atopic dermatitis. Previous work by the research group has shown that reduced levels are seen in the mucosal lymphocytes of active UC. Thus, suggesting perhaps, the Th2 pathway is not the response in UC. I will look at the levels of TSLP in mucosal biopsies of active UC, quiescent UC, and normal colonic biopsies to confirm this response.

1.1.2.3 Environmental factors

There seems to be little evidence to support that dietary factors influence the development of IBD. However, given the increased rates of IBD in western societies this is still hypothesized to play a role. Emigration from an area of relatively low prevalence of IBD such as Asia, to an area of high prevalence in Northern Europe, the populations with previously lower rates of IBD are increasing(*27*), suggesting a strong environmental factor, of which the western diet is one. New studies in mouse models have implicated emulsifiers in the diet, which is particularly found in the western diet, as a cause for inflammatory cascade induction within the gut. The concept is that these agents disrupt the host microbiota interaction promoting low-grade inflammation, through the promotion of the metabolic syndrome and adiposity formation. There is a suggestion that these agents may be

contributing more generally to the western societal problem of obesity and chronic inflammatory disorders(*28, 29*).

Another dietary factor includes vitamin D. Vitamin D is well known to be essential in the functioning of the immune system, and deficiency has been implicated in several immune mediated diseases(*30-32*). With regard to inflammatory bowel disease, vitamin D has been shown to play a role in the maintenance of the intestinal epithelial barrier(*33, 34*), the regulatory immune system at the level of the intestinal mucosa(*35, 36*), and the fine balance of host intestinal microbiota(*37*). Vitamin deficiency has been proposed as a potential cause of inflammatory bowel disease through these mechanisms(*34, 38-40*), however vitamin D therapy is not known to be effective in the treatment of IBD.

Smoking is a well-known factor influencing the development and course of IBD. It may increase the risk of CD and is associated with a more aggressive phenotype with less response to drug treatment and increased surgical rates and complications. Conversely it is protective in UC(*41*). In siblings with the genetic susceptibility to develop IBD, the smokers tended to develop CD whereas the non-smokers UC(*42*).

The large American nurses' health studies I and II have also shown some interesting environmental factors influencing IBD. These prospective studies followed 121700 and 116000 nurses respectively across 11 states, from 1976 and 1989, between the ages of 25 and 42(*43, 44*). This study confirmed the risk factor of smoking, but also showed geographic variations across the US, supporting the concept of vitamin D deficiency due to lack of UV exposure as a possible causative risk factor(*43*). Other risk factors exposed were the use of non-steroidal anti-inflammatory drugs and the oral contraceptive pill for the onset of Crohn's disease, and the hormone replacement therapy in postmenopausal women in the onset of ulcerative colitis(*43*). Dietary intake of fibre and certain fatty acids was also associated with the development of IBD(*43*).

Other factors involved in the environment role in IBD development are non-steroidal anti-inflammatory use(*45*), childhood appendicectomy has a protective influence(*46, 47*), and measles virus(*48*).

1.1.2.4 The Gut Microbiota and Dysbiosis

The colonisation of the gut starts to occur at birth, and the diversity increases rapidly to develop a stable microbiome(*49*). The gut microbiota consists of a complex balance or organisms that live in our digestive tract. Bacteria make up to 60% of faecal matter and up to 1000 different species of bacteria exist in our gut, although 99% of them are confined to 30-40 species(*50, 51*), the commonest of which are *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteriodetes(52).* The symbiotic relationship between human and the gut microbiome is essential. By providing a suitable environment for the gut microbiota to flourish, the microbiota assists us through fermentation of carbohydrates to short chain fatty acids, synthesis of vitamins, such as vitamin K, promotion and control of intestinal epithelial cell growth, and important protective functions against pathological microbials(*53, 54*). The microbiota also have a continuous effect on the host's intestinal and systemic immune systems.

The microbiota varies significantly between individuals and can fluctuate within a single individual over time. Factors such as host genetics, diet, antibiotic use, the external environment, and intestinal inflammation all play a role in influencing the makeup of the gut microbiome, and host immune response to individual species. The recent advances in sequencing have enabled research into the microbiome, and the influences it may have in health and disease, as such it is now widely agreed that the dysregulation of the gut microbiota plays an important role in the pathogenesis of IBD.

Several animal studies have shown that the gut bacterial colonisation is critical in the development of IBD(*55*). Crohn's Disease and ulcerative colitis do preferentially occur in the areas of bowel with the highest concentrations of microbiota, namely the colon and distal ileum, and studies have shown that the diversity of the microbiota is depleted in IBD, and that the distribution of the bacteria moves from favouring *Firmicutes* and *Bacteriodetes,* towards an expansion in the *Actinobacteria* and *Proteobacteria* subsets of microbiota(*56*). Twin studies have been done in which twin pairs either both affected or only one affected with Crohn's disease or UC had their gut microbiota sequenced. These confirmed reduced microbiota diversity in the subjects with IBD(*57*). As yet no specific causative agent has been identified within the microbiota however there is interest in studies which have shown that the firmicute *Faecalibacterium prausnitzii* is consistently depleted in the mucosal samples of

IBD patients(*57, 58*), and low concentrations of *F. prausnitzii* in the ileum can predict increased risk for recurrence of ileal Crohn's Disease(*58*). The specific and varied diversity and colonisation profiles are still not known to be either primary causative or secondary responses to the inflammation seen in IBD.

Invasive *Escherichia coli* is found in much greater abundance in inflamed mucosa of patients with active ileal Crohn's disease when compared to normal healthy patients and even patients with colonic disease(*59-61*). *E. Coli* invades the epithelium and replicates in macrophages and causes inflammation by secreting large quantities of tumour necrosis factor.

The mycobacteria, *Mycobacterium Avium Paratuberculosis*, has been implicated as a possible causative agent, as it causes IBD like intestinal chronic granulomatous inflammation in ruminants, and has been found in varying concentrations in frequencies in patients with Crohn's disease(*62*). However, antituberculous triple therapy has proven ineffective treatment in induction and maintenance of remission in IBD(*63*).

Host genetic defects can cause impairment of microbial killing and therefore increasing the exposure of the intestinal barrier to potential pathogenic bacteria. For example defects in the transcription factor TCF4, which controls downstream expression of genes responsible for Paneth cell maturation and differentiation has been associated with Crohn's disease(*64*). This in turn reduces the secretion of α-defensins by Paneth cells which are responsible for protection against *E. Coli*(*64*).

The potential for the use of faecal intestinal microbiota transplantation has been explored in the treatment of IBD and will be discussed in section 1.1.5.5.

1.1.3 Clinical features and diagnosis of Crohn's disease

IBD can present at any age but most commonly presents between the ages of 16 and 30. Overall it affects both sexes equally, although there has been studies suggesting a female predominance in CD(*65*). There is an additional peak seen during the sixth and seventh decades of life, but this is clearer for UC. CD is characterised typically by transmural inflammation of the bowel wall and can affect any part of the GI tract (unlike UC). The diseased bowel is often not confluent leading to so called skip lesions. The site and severity of these chronic inflammatory lesions can lead to a variety of phenotypes, such as stricturing and fibrosis, or penetrating disease leading to perforation, abscess or fistulising disease. These phenotypes are not constant among individual patients, and patients with one phenotype can develop another. The most common site for CD is the terminal ileum but other common sites are diffuse small bowel disease, colonic disease, and perianal disease. The latter of which confers a worse prognosis. Isolated gastroduodenal or oesophageal disease is rare.

Symptoms often present insidiously, and in a relapsing pattern. They may occur for several years before a diagnosis is sought and made. The common symptoms are diarrhoea, abdominal pain, weight loss, and constitutional symptoms resulting from inflammation, malabsorption of nutrients and anaemia. Also, other symptoms as a result of fistulising or stricturing disease may be present. The clinical features are wide ranging and are dependent on the area and extent of the involved bowel.

No single laboratory test can be used to diagnose CD. Commonly CRP and ESR are high during periods of inflammation. Anaemia may be present due to malnutrition, vitamin deficiencies and anaemia of chronic disease, and leucocytosis and thrombocytosis are common. Various serological markers have been associated with CD, including P-ANCA in Crohn's Colitis and UC, but these tests have limited diagnostic accuracy and clinical utility.

Assessing the stool is used to look for alternative infective aetiology during a flare including *clostridium difficile* and other gut pathogens. More recently the use of stool to measure faecal calprotectin has been developed especially in paediatrics(*66-68*). Its high sensitivity for gut inflammation makes it a useful screening tool for IBD and assess for relapsing

disease, but alternative causes of inflammation reduce its specificity as a diagnostic marker(*69*).

Imaging and endoscopy of the bowel remains the most powerful diagnostic tool with colonoscopy being the mainstay of diagnosis and assessment of both CD and UC as it allows both direct visualisation of the mucosa and histological sampling. Classic findings in CD are that of erythema, apthoid ulceration, cobblestone granularity, and occasionally strictures. Endoscopic and histological appearances cannot always reliably distinguish CD from UC when confined to the colon, however skip lesions, ileal ulceration, and fistulae if present are all associated with CD.

The small bowel can be difficult to access for tissue sampling although enteroscopy is possible in specialised centres. Capsule endoscopy allows direct visualisation but is contraindicated in structuring disease. Radiological imaging using CT/MRI enterography/enteroclysis or Small bowel ultrasound allows detailed imaging of the bowel assessing extent of disease and intestinal complications such as abscess and fistulae formation.

1.1.4 Clinical features and diagnosis of Ulcerative Colitis

Ulcerative Colitis can also occur at any age but again commonly presents in early adulthood, with a peak again in the sixth and seventh decades of life. UC tends to have a much more homogenous presentation with bloody diarrhoea the hallmark feature. This is due to the inflammation being confined to the large bowel. Other common symptoms may include tenesmus and colicky abdominal pain. The rectum is virtually always involved, and inflammation extends in a confluent manner proximally through the colon for a variable distance. Rectal sparing can occur but is usually a result of treatment. It can involve the rectum alone (Ulcerative Proctitis) or extend to a variable extent proximally. If the whole colon is involved this is termed pancolitis.

UC can present acutely causing severe disease. The first presentation can often be the worst (as it has been untreated). The mortality for UC was historically high up to 50%(*70*) in the 1950s. However advances in medical therapy especial cortisone has drastically reduced the mortality of UC(*70*). Acute severe UC is still a potentially life-threatening disease. UC also

classically follows a relapsing remitting course, but a minority of patients follow a chronic insidious course. Approximately 10-20% of patients with UC will have a colectomy in their lifetime, but this has been reduced from 50% in the prebiologic treatment era(*71-73*). This Figure is likely to have reduced further with the earlier and more aggressive use of biological therapies over recent years.

Investigation for UC is much like that of CD. Laboratory finding may be similar with iron deficiency anaemia and raised CRP. Stool studies are often done to rule out concomitant infective disorders especially *C.Difficile*. Serological markers have been studied and P-ANCA is again a commonly associated marker predicting a more aggressive course that is more likely to be disease refractory requiring colectomy. Its use as a diagnostic marker remains uncertain and as it can also be present in CD, it has a limit use to distinguish UC from CD. There is however suggestions in studies that it does have a preponderance towards UC(*74*).

The diagnosis of UC relies on a combination of clinical presentation along with the endoscopic appearances and histological appearances of the colon. Histology alone cannot diagnose UC.

1.1.5 Treatment of inflammatory bowel disease

There are two specific aims that are thought of when using drug therapy to treat inflammatory bowel disease. Firstly, drug therapy is used to induce remission of active inflammatory disease (induction). Secondly, drug therapy is used to maintain that remission of controlled disease (maintenance). There are 4 groups of drugs used in IBD. These are 5-Aminosalicylates (5-ASA), corticosteroids, immunomodulators, and biologics which currently include anti-TNF agents and the newer anti-integrin agent vedolizumab. Other drugs are also used less commonly such as antimicrobials and probiotics, and there is debate over the use of faecal transplantation therapy, but there is no clear evidence for this.

1.1.5.1 5-Aminosalysilic Acid

5-ASA treatment is used mainly in ulcerative colitis, as first line therapy for mild to moderate disease. Evidence shows it can effectively induce remission in mild to moderate UC compared to placebo(*75*), but are no more effective than sulphasalazine(*76*). Topical and oral forms of 5-ASA are available, but there is no evidence that one formulation is superior

to another(*75*). There is a dose response with 5-ASA, and this has been shown by the inverse correlation seen between disease severity and mucosal 5-ASA concentration(*77*). 5-ASA are also used as chemoprotective agents, to reduce the risk of dysplasia and colorectal cancer in patients with UC. A systematic review showed that 5-ASA treatment does have a protective effect in the development of colorectal cancer(*78*).

The mechanism of action of 5-ASA drugs is not clear. There is evidence that targeting of peroxidase proliferator-activated receptor-γ (PPAR-γ) by 5-ASA increasing its expression and its anti-inflammatory effects(*79*). 5-ASA are also thought to be scavengers of oxygen free radicals(*80*). Other mechanisms hypothesised are prostaglandin inhibition(*81*), IL-1 inhibition(*82*), and antagonism of neutrophil chemotaxis(*83*).

Other drugs which work on the same pathway are the thiazolidinedione group. These include Pioglitizone and Rosiglitizone which are used in the treatment of diabetes mellitus. They control the gene involved in lipid and insulin metabolism by binding to the peroxidase proliferator-activated receptors (PPARs) and may have anti-inflammatory effects(*84*).

These agents have not shown to be effective in IBD(*85*), but it does suggest this pathway could be targeted in the future for novel agents.

1.1.5.2 Immunomodulators

The most commonly used immunomodulator used in IBD are thiopurines in the form of azathioprine and 6-mercaptopurine (6-MP).

Thiopurines are proven to be effective in IBD. 55% of patients achieve maintenance of steroid-free mucosal healing and symptomatic remission after initial induction therapy for up to 5 years(*86*). However, thiopurines are of limited use in induction of remission and are reserved for the long-term use of disease maintenance. They are now being used earlier in disease, and advances in metabolite monitoring, means that both dose optimisation and toxicity avoidance can make them a more efficacious drug group.

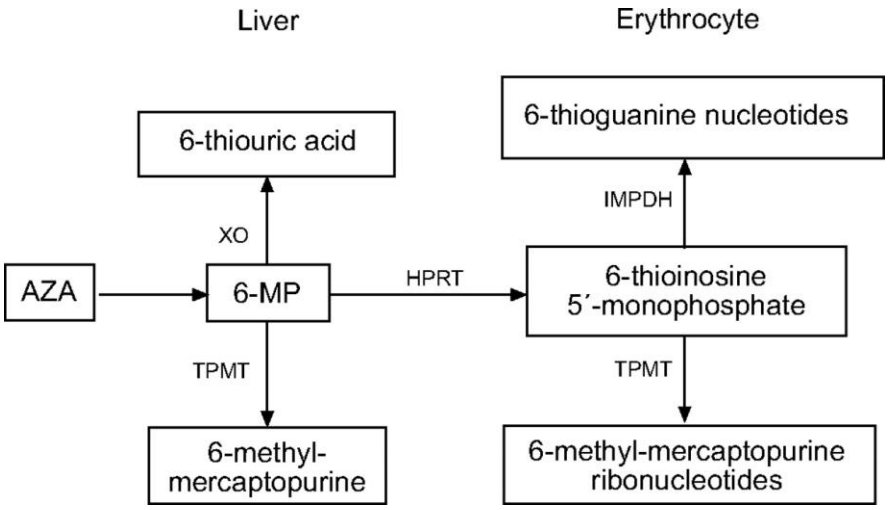


Figure 1. Azathioprine metabolism pathway (87)

Azathioprine is the pro-drug of 6-MP and is converted to 6-MP in the liver. Both drugs have no intrinsic activity, therefore both drugs are metabolised to form 6-Thioguanine nucleotides (6TGN), the active metabolites, and other metabolites such as 6-Methyl Mercaptopurine (6MMP), which are associated with toxic side effects. 6TGNs inhibit enzymes required for DNA synthesis, and therefore its main action is on proliferating cells, such as T-cells and B-cells of the immune system(*88*).

Intracellular uptake of 6MP occurs, and 3 enzymes metabolise it to its metabolites. Thiopurine methyltransferase (TPMT) methylates 6MP to form 6MMP. Xanthine Oxidase (XO) metabolises it to 6-thiouric acid (6TUA). Hypoxanthine phosphoribosyl transferase (HPRT) produces 6-thioinosine monophosphate (6TIMP), which is further metabolised by inosine monophosphate dehydrogenase (IMPD) into 6-thioxanthosine monophosphate (6TXMP), and ultimately metabolised to 6-thioguanine-monophosphate (6TGMP), -diphosphate (6TGDP) and -triphosphate (6TGTP), together the so-called 6-Thioguanine nucleotides (6TGN)(*89, 90*). The 6TGNs act as purine antagonists and induce immunosuppressive effects by inhibiting DNA and RNA synthesis(*88*).

Other immunomodulators are used less commonly including Methotrexate, Tacrolimus, and Cyclosporine.

1.1.5.3 Biologics

The three anti-TNFα biological therapies currently licensed for use in the United Kingdom. These are Infliximab, Adalimumab, and Golimumab. However, Golimumab is only licensed

for the treatment of UC. The alternative biological therapy approved for use in IBD is the anti-integrin monoclonal antibody Vedolizumab.

Infliximab is a chimeric murine monoclonal antibody. Adalimumab and Golimumab are recombinant human IgG1 monoclonal antibodies. The exact mechanism of action is unclear, however they bind to soluble free, and transmembrane TNFα with high affinity and are highly specific(*91*). In doing so anti-TNFs are thought to neutralise the biological activity of TNFα, by inhibiting the effective binding of TNFα to its receptor(*92*). The activities attributed TNFα include the production of proinflammatory cytokines such as interleukin-1β and interleukin-6, and it has been shown that these cytokines are reduced following infusions of anti-TNF(*93*). Vos et al have shown that the superiority of combination infliximab and azathioprine therapy over monotherapy, may be due to the regulatory macrophages that are involved in mucosal healing(*94*).

Several trials have shown Infliximab and Adalimumab to be effective treatments for CD, and are used as both induction agents and for maintenance of remission. The ACCENT I and II studies have shown that Infliximab is an effective induction of remission agent in CD and can effectively treat the complications of CD such as fistulae(*95-97*). Moreover, the SONIC trial has shown superiority of combination Infliximab and thiopurine therapy over Infliximab or Thiopurine monotherapies in treatment of CD(*98*). The CHARM study showed that Adalimumab was significantly more effective than placebo in maintaining remission in moderate to severe CD(*99*).

There is also evidence for the use of anti-TNF therapy in UC. The CONSTRUCT trial has shown that infliximab is as effective at inducing remission as cyclosporine in acute severe ulcerative colitis(*100*), and is a recognised rescue therapy in this setting. Infliximab was shown to be superior to placebo in the ACT 1 and 2 trials, in both induction of remission and maintenance of remission at week 30(*101, 102*). Adalimumab has been shown to be and effective induction agent for moderate to severe active UC(*91*).

Van den brink et al have shown that development of anti-drug antibodies against infliximab and adalimumab is a significant cause in 77% of people who lose response to these anti-TNF therapies(*103*). They also show that the introduction of immunosuppressive therapy to

suppress the formation of anti-drug antibodies is associated with better clinical and histological outcomes(*103*).

The PURSUIT and GO-COLITIS trials have shown Golimumab be an effective subcutaneous treatment for both induction of clinical and endoscopic remission and mucosal healing in UC as well as the maintenance of remission when compared to placebo(*104, 105*).

Vedolizumab is a gut specific integrin α4β7 blocker, and is the newest licenced drug for the treatment of IBD. Vedolizumab has been shown to be an effective and safe treatment for both induction and maintenance of remission in patients with moderate to severe UC at week 52 from the GEMINI-1 study group(*106*). It has also been found to be an effective treatment in Crohn's disease. However, patients were more likely to be in clinical remission at week 6, the vedolizumab group did not meet the end point of CDAI-100 response(*107*). Also a further trial showed that vedolizumab was not more effective than placebo in patients who had previously failed anti-TNF therapy by week 6, although there was a statistical benefit by week 10, suggesting vedolizumab may not be as effective in Crohn's disease as it is in UC(*107, 108*).

There are newer agents also in current clinical trials that will hopefully soon be available for use in IBD.

Trials using Ustekinumab a anti IL12/23 monoclonal antibody(*109*), Etrolizumab a monoclonal antibody to the integrin subunit β7(*110, 111*), and Certolizumab pegol a pegylated antiTNFα monoclonal antibody(*112*), have all shown modest efficacy in the induction of remission in IBD patients who have failed antiTNF biological therapy when compared to placebo.

1.1.5.4 Elemental diet and Probiotics

There is no indication for the use of elemental diet for the treatment of UC, however it may be useful in small bowel CD(*113*). It is often used in children and has been shown to be an effective strategy for inducing remission small bowel CD(*114-116*), but the evidence is not so robust in large bowel CD. The evidence is less clear for adult CD and there is little evidence for the use of elemental diet as maintenance therapy(*117*).

Probiotic therapy can be administered as single organism or a mixture of organisms, with the aim to alter the gut microbiome towards that of a normal individual. Agents used include VSL#3, *E-coli Nissile*, and *bifidobacterium*(*118*). There is limited evidence for the treatment of UC with VSL#3 as an induction therapy(*67, 119*), but also in the maintenance of remission of UC(*67*). *E-coli Nissile* has been shown to be an effective alternative therapy to 5-ASA therapy in maintenance of remission of UC(*120*). There is no evidence for the use of probiotics in CD(*121*).

1.1.5.5 Faecal Microbiota Transplantation

The idea that whole microbiota dysregulation rather than single organism variation is more important for the development of inflammatory bowel disease has been established in research. Faecal microbiota transplantation has been shown to be effective in the treatment of refractory *clostridium difficile* infection(*122*) and there have been recent studies looking into the role it could play in the treatment of inflammatory bowel disease, particularly ulcerative colitis.

Rossen et al found that in 37 patients with mild to moderately active ulcerative colitis randomised to receive either nasoduodenal administration of faeces or placebo there was no statistical difference in the primary endpoints of clinical and endoscopic remission between the groups at 12 weeks follow up. However distinct microbiome signals could be seen in the responders(*123*).

Moayyedi et al also did show a statistical difference between the treatment and placebo group in meeting the primary endpoint of a drop in the Mayo score of 2 or more with an endoscopic mayo score of 0 (24% vs 5%). They also showed that 7 of the 9 patients who achieved remission received the faeces from a single donor(*124*). This suggests that analysis the microbiome of the donor could significantly improve outcomes using this treatment.

The most recent study in 85 patients with ulcerative colitis randomised between placebo and transplantation has shown that intensive multi-donor faecal microbiota transplantation is effective at inducing clinical remission, and improved endoscopic appearances. However the outcomes of endoscopic and histological remission were not met(*125*).

Overall in 4 randomised control trails the results have been contradictory, but there is a signal that faecal microbiota transplantation may be an effective treatment for ulcerative

colitis either using multiple donors or with targeted donors using microbiome sequencing. Specific protocols and further research in this field is still required. It is also not known if this therapy will be an effective maintenance treatment(*126*).

1.1.5.6 Surgery

Surgery remains an important option in the management of both UC and CD.

Up to 30% of patients with UC will ultimately have a colectomy(*127*). Subtotal colectomy followed by ileoanal pouch formation or panproctocolectomy with permanent ileostomy are two surgical options and patients need to be selected carefully for each procedure. The indications for surgery in UC are, poorly controlled disease not responding to medical therapy whether chronic active or acute severe disease, UC complicated by toxic megacolon or perforation, and dysplasia or carcinoma.

Up to 75% of patients with a 10 year history CD will undergo some form of surgery(*128*). Despite advances in medical therapy the need for surgery has not reduced significantly, with over 50% of Crohn's patients undergoing surgery during their lifetime(*129, 130*). The indications for surgery in CD is not clearly defined as it is in UC, and decisions need to be made carefully with a multidisciplinary approach. Decisions are based on disease location and extent, disease complicated by fistulation, stenosis or sepsis, and preservation of bowel length.

1.1.6 Clinical scoring systems

There are several scoring systems that can be used in UC and CD. The Truelove and Witt criteria are used to assess the initial clinical severity of UC and have been developed in to the day 3/Travis criteria to also assess response to steroid therapy and need for rescue therapy in acute severe colitis(*5*).

Activity	Mild	Moderate	Severe
Number of bloody stools per day (*n*)	<4	4–6	>6
Temperature (°C)	Afebrile	Intermediate	>37.8
Heart rate (beats per minute)	Normal	Intermediate	>90
Haemoglobin (g/dl)	>11	10.5–11	<10.5
Erythrocyte sedimentation rate (mm/h)	<20	20–30	>30

Table 2. Truelove and Witt criterial for severity of UC

The Mayo disease activity index for UC also incorporates endoscopic findings, and with the focus on mucosal healing as a disease endpoint is becoming a popular alternative scoring system. A score of 0-2 indicates remission, 3-5 mild disease activity, 6-10 moderate activity, and 11-12 severely active disease. The Mayo activity index is summarised in table 2(*131*).

Variables	Score 0	Score 1	Score 2	Score 3
Number of stools per day	Normal	1-2 stools more	3-4 stools more	5 or more stools more
Rectal bleeding	None	Streaks of blood less than half the time	Obvious with blood most of the time	Blood alone
Findings at endoscopy	Normal or inactive disease	Erythema, decreased vascular pattern, mild friability	Marked erythema, absent vascular pattern, friability, erosions	Spontaneous bleeding, ulceration
Physicians Global assessment	Normal	Mild Disease	Moderate disease	Severe disease

Table 3. The Mayo disease activity index (Remission 0-2, Mild 3-5, Moderate 6-10, Severe 11-12)

In Crohn's disease the gold standard tool to quantify symptoms and severity of Crohn's disease is the Crohn's disease activity index (CDAI). The CDAI is the most common way that clinical trials assess and define clinical response and remission of CD to new therapies but is cumbersome to use in clinical practice. *The CDAI is often criticised for its lack of endoscopic factors, and the fact it does not take quality of life assessment in to its calculation.* The components of the CDAI are summarised in table 3(*132*).

Variables	**Weighting**
Number of liquid stools per day over the last 7 days	x 2
Abdominal pain over past 7 days (Graded 0-3 in severity)	X 5
Subjective general wellbeing over past 7 days (Graded 0 (well)-4 (terrible))	X 30
Presence of complications* (Score 1 for each complication)	x 20
Taking antidiarrhoeal or opiates for diarrhoea (Score 1)	x 30
Presence of an abdominal mass (0 as none, 2 as questionable, 5 as definite)	x 10
Haematocrit of <0.47 in men and <0.42 in women (Score 1)	x 6
Percentage deviation from standard weight	x 1

*Joint pains (arthralgia) or frank arthritis.
*inflammation of the iris or uveitis.
*presence of erythema nodosum pyoderma gangrenosum or aphthous ulcers.
*anal fissures fistulae or abscesses
*other fistulae
*fever during the previous week.

Table 4. The Crohn's disease activity index (Remission <150, Severe CD >450, Response drop by 70)

A simpler clinical scoring system was devised in 1980 by Harvey R, and Bradshaw M, the so-called Harvey Bradshaw Index(*133*) (HBI). This system is easier to use than the CDAI for data collection purposes as it only consists of clinical parameters. The HBI is summarised in table 4.

Variables	**Score**
Number of liquid stools per day	N
Abdominal pain	0 – none 1 - mild 2 - moderate 3 - severe
Subjective general wellbeing over past 7 days	0 - Very well 1 - Slightly below par 2 - Poor 3 - Very Poor 4 - Terrible
Presence of complications as per CDAI*	Score 1 for each
Abdominal mass	0 - none 1 - dubious 2 - definite 3 - definite and tender

Table 5. Harvey Bradshaw Index (Remission <5, Mild CD 5-7, Moderate CD 8-16, Severe CD >16)

1.2 MicroRNA(*134*)

1.2.1 MicroRNA Overview

MicroRNA (miRNA) was first discovered in 1993. Lin-4 small RNA, discovered by Lee et al, was the first microRNA to be discovered and was shown to regulate the development of *C Elegans(135, 136)*. Let-7 was a second small RNA discovered in *C.Elegans,* and has subsequently been found as a human microRNA(*137*). The Let-7 family of microRNAs is found in most animals. The high degree of conservation of let-7 miRNA across many species suggests a significance role for miRNAs within immune regulation(*137, 138*).

MiRNAs are short 19-23 nucleotide segments of single-stranded RNA. Along with small interfering RNAs they make up part of the small noncoding regulatory RNA group. They are transcribed from intronic or intergenic regions of DNA by RNA Polymerase 2 as pri-miRNA(*139, 140*). A single pri-miRNA may contain up to six miRNA precursors. The pri-miRNA binds with DiGeorge Syndrome Critical Region 8 (DGR8), a nuclear protein, and the enzyme Drosha, to form a complex which is then processed to form a ≈70 base pair precursor RNA (pre-miRNA) (*141, 142*). Exportin 5 then mobilises the pre-miRNA to the cytoplasm(*143*) where RNase III endonuclease Dicer cleaves it to form a mature miRNA:miRNA Duplex(*144*). The active strand of the mature miRNA is incorporated into the RNA induced silencing complex (RISC). RISC is a multi-protein complex that incorporates one miRNA strand. RISC uses the miRNA as a template for recognizing complementary mRNA. When it finds a complementary strand, it activates RNase and cleaves or represses the RNA(*145, 146*). This process is an important part of regulation of gene expression by miRNAs. (Figure 2.)

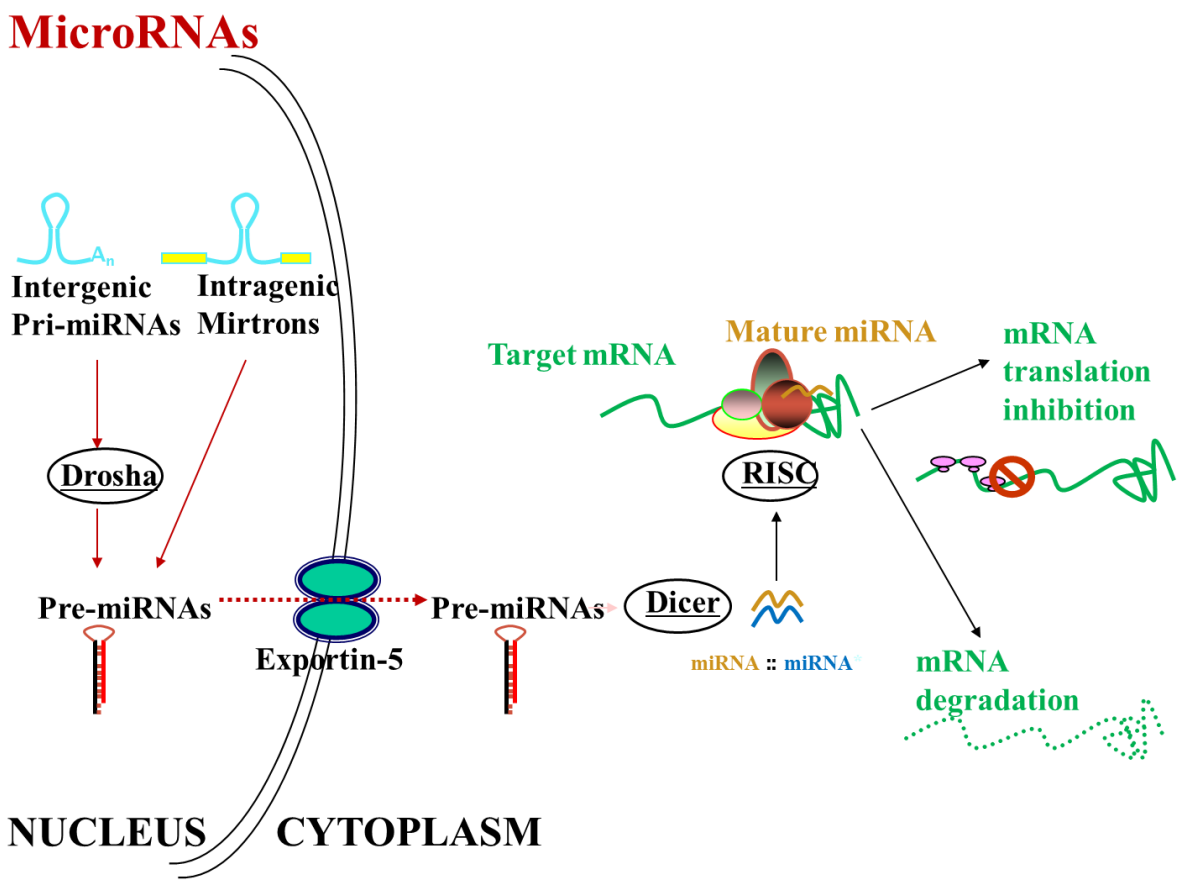


Figure 2. Pathway for the synthesis of microRNAs

The miRNA binds to complementary sequences of 3-Untranslated regions(3-UTR) on messenger RNA (mRNA) to either repress protein production by inhibiting translation or induce mRNA degradation(*147*). The miRNA binding does not have to be 100% complementary to the 3-UTR of the mRNA it is repressing and therefore one miRNA can target many mRNAs, and a single mRNA can be a target for many miRNA(*148*).

Emerging evidence is now showing that miRNAs are of physiological importance and necessary for the regulation of normal cellular homeostasis and development. Complete miRNA knockout in the developing mouse has shown to be fatal with in utero attesting to this(*149*). Further organ specific knockout studies have resulted in distinctive abnormal phenotypes(*150*). In particular complete miRNA knockout in the intestinal epithelium results in an abnormal intestinal epithelium with expanded crypts, increased apoptosis with lymphocyte and neutrophil infiltration(*151*). This resulted in increased epithelial permeability and decreased barrier function as assessed using labelled lactulose and mannitol(*151*).

miRNAs are clustered on chromosomes, often proximal to gene clusters and are thus co expressed(*152, 153*). This suggests miRNA expression may act within a coordinated network to control a target gene or network of genes. One of the most extensively studied networks is the 17/92 cluster, comprising miRNAs 17, 18a, 19a, 20a 19b-1 and 92a-1. This cluster also has 2 paralogues in the human genome; 106b/25 comprising of miRNAs 106b, 25, 93 and 106a/363 comprising miRNAs 106a, 18b, 20b, 19-b-2, 92-a2 and 363(*154*). This network has been shown to regulate normal development, cell cycle and proliferation and to be dysregulated in several human diseases including solid organ and haematological malignancies(*154*) (Table 5). As can be seen there are several target genes in each pathway which are controlled by this miRNA cluster. Thus, a complex picture emerges of miRNAs acting in a tightly coordinated manner, functioning as a post–transcriptional homeostatic switches regulating protein production. A further detailed review of this cluster is proved by Mogilyansky et al(*154*).

Function	Genes involved
Cell Death	PTEN , BCL2L11, MEF2A, IRF1, Fasl
Cell Cycle Arrest	p21, p57
Cell Cycle Regulation	ESF1, ESF2, ESF3
Heart/Lung Development	Isl-1, Tbx-1, STAT3, MAPK14
Hypoxia	HIF-1α
WNT Pathway	ZBTB33
Angiogenesis	TSP-1, CTGF
Immune Regulation	STAT2
Cell Proliferation	SMAD2, SMAD4, ESR1, FOSL2, NR3C1
Unknown function	CHD2, SUZ12, TALI, SP1, USF2

Table 6. Genes controlled by the 17/92 microRNA cluster.

Bioinformatics approaches show that an individual miRNA can have a large number of binding sites (up to 2000). The degree of complementarity between the miRNA and target mRNA plays an important role in determining the influence the miRNA has on repressing mRNA translation or degradation. The mutation of an mRNA 3'UTR could provide new binding sites for different miRNAs or inhibit the effect of the normal regulatory miRNAs;

however other factors also play a role in the interaction between miRNA and mRNA. The secondary structure and thermodynamic properties of mRNA also plays a pivotal role in the ability of miRNA to access their target sequence(*155-159*). Kertesz et al showed that mutations which disrupted the site accessibility of the target mRNA sequences, led to a reduction in the repression effect of miRNA on the mRNA similar to that of mutations that changed sequence complementation(*155*). RNA binding proteins (RBPs) play an important role in the regulation of transcript decay. Computational analysis has supported the hypothesis that RBPs work with miRNAs to enhance mRNA degradation by moving miRNA target sites to more accessible regions(*160*).

The control of microRNA transcription is thought to be internally controlled by its location to the gene it is suppressing. Although microRNAs may target many genes in order to supress gene translation, their effect may be targeted to specific genes as they are translated concomitantly with the target gene. It has been found the microRNAs are coded for within the non-coding sequence of the gene that they target and are therefore are co transcribed. The effects of both inflammation and treatments may affect the transcription of the microRNA and target gene, disrupting the balance leading to over or under expression.

The number of binding sites and the complex interactions at miRNAs binding sites have important implications for the function of miRNAs in the context of the control of inflammation. Depending on the cell type, mRNA expression and the presence (or absence) of other miRNAs, in-theory at least, an individual miRNA may have very different effects on regulation of a specific inflammatory pathway. This has not been explored in any detail in the context of IBD as yet and will require carefully designed experiments in well characterised patients.

1.2.2 MicroRNA in IBD

The role of miRNAs in IBD is unclear with little published data. UC and CD appear to have unique miRNA profiles in their target tissue. Consistent with the emerging genetics story, some differentially expressed miRNAs are common to both diseases, whilst others may be unique to either the disease or the tissue type. Although most of these are unique to inflammatory bowel disease, there are some that are common to other autoimmune

conditions(*161*). Understanding the role of miRNAs in IBD will allow a deeper understanding of disease mechanisms, but they may have a role as new diagnostic and prognostic biomarkers, in the identification of new therapeutic targets, and as therapeutic agents themselves.

Several groups have published analyses of miRNA expression in IBD patients from both tissue and serum using a microarray approach. To date, 5 groups have published studies, which have previously been summarised(*153, 162*). These studies produce conflicting results and are difficult to compare due to heterogeneous study populations. Sample sizes vary considerable for the microarray analyses from 2 to 128 patients(*163, 164*). Many studies do not control for concomitant medication use, the inflammatory status of the biopsies nor the location from which the samples were taken all of which may influence the results. Serum miRNA profiles may reflect non-IBD related inflammatory processes in patients. Data from whole tissue biopsies offers no insights into which specific cells are responsible for miRNA production, or how miRNAs may influence the interactions between different elements of the gut mucosa and immune system. This data must therefore be interpreted with caution and provides little insight into the role of miRNAs in IBD pathogenesis.

Genome wide association studies have identified new pathways as being important in the pathogenesis of CD including autophagy, however little progress has been made with understanding the functional significance of many of these markers, particularly those found in non-coding regions. One such region containing polymorphisms is that of the IRGM protein sequence expression coding region(*165-167*). Overexpression of miR-196 has been shown to be associated with the down regulation of the protective variant of the IRGM coding protein (c.313T)(*168*), leading to the loss of control of the replication of E-coli (a Crohn's disease associated intracellular bacteria) due to the dysfunction of IRGM regulated autophagy(*168*). This has shown how miRNA-based pathways are critical to the normal homeostasis in the bowel and that alterations in the miRNA expression can influence the disease process.

Of the other studies published so far, miR-192 also has a potential mechanistic function of a differentially expressed miRNA in IBD. Wu et al has shown miR-192 to be reduced in colonic biopsies from active UC patients. The expression of the pro inflammatory chemokine, Macrophage inflammatory peptide 2α (MIP 2α), was also shown to be increased in these

biopsies. Reporter assays in cell culture subsequently suggested that miR-192 regulated MIP-2α expression(*169*). More recently miR-141 has been shown to target CXCL12β therapeutically. CXCL12β is involved in the up regulation of total-CXC12 in intestinal inflammation. This miRNA:mRNA targeting alters leukocyte trafficking during the inflammatory process of colonic CD by influencing immune cell recruitment(*170*).

Several other specific miRNAs have been implicated as having a role to play in the pathogenesis of IBD, for example, miR-31 and miR-146a have been shown to be expressed specifically in the inflamed tissue of both UC and CD(*171*). A further study using miRNA microarray and real time qPCR has shown the overexpression of miR-23b, miR-106,and miR-191 in active CD colitis compared to healthy normal subjects and miR-16, miR-21, miR-223, and miR-594 in active ileal CD disease compared to healthy normal subjects(*172*). This study also showed down regulation of miR-19b and miR-629 in active colonic CD disease compared to healthy normal subjects. The only miRNA found to have been down regulated in active ileal CD (miR-422b) was not confirmed by RTqPCR(*172*). The same Group also showed up regulation of miR-21, miR-23a, miR-126 and miR-195 to be up regulated in active Ulcerative colitis, and miR-192 was down regulated in active Ulcerative Colitis(*169*).

MiR-124 has been shown to be down regulated in the colonic mucosa of paediatric patients with active Ulcerative Colitis, when compared to inactive colonic mucosal samples. miR-124 regulates the expression of Signal transducer and activator of transcription 3 (STAT3) and its under expression could result in overexpression of STAT3 and therefore increased inflammation(*173*).

MiR-155 is an important miRNA in the regulation of the immune system. Furthermore, it appears that it plays a major role in the development of $CD4^+$ T Cells. Although this may have a protective role in the context of infections, it may be detrimental in the context of autoimmune disease(*174*). miR-155 has been shown to skew the lineage between Th1 and Th2 pathways by repressing SOCS1(*175*). miR-155 has been specifically shown to be up regulated in patients with severe UC(*164*).

1.2.3 MicroRNA in other disease

1.2.3.1 Cancer

There is evidence that miRNAs may have a significant role in tumour biology. MiRNA abnormalities in cancer were first described in B-cell Chronic Lymphocytic Leukaemia, with down regulation of miR-15 and miR-16 in 2002(*176*). MiRNA can be overexpressed and thus produce an inhibitory effect on tumour suppressor genes, or cell apoptosis genes. Likewise under expression of miRNAs may cause overexpression of oncogenes or other pro-oncotic genes(*177*). Interestingly, miRNAs are dysregulated in inflammatory conditions that are associated with increased risk of cancer including the up regulation of miR-17, miR-122, and let-7e, all of which target the TP53 pathway, in IBD-associated colorectal cancer(*178*).

1.2.3.2 Autoimmune disease

As we begin to understand the importance of miRNAs in immune regulation, studies have shown miRNA dysregulation to be associated with many of the other autoimmune pathologies. Unique miRNA profiles can be seen in various autoimmune diseases, such as psoriasis, SLE, Rheumatoid Arthritis, and Eczema(*161*). Some miRNAs are common across diseases whereas some are tissue or disease specific (Table 6). This may well reflect the experience in GWAS studies where an individual SNP may be associated with more than one disease(*179*).

Disease	Up regulated microRNAs	Down regulated microRNAs
Rheumatoid arthritis	miR-16, miR-132, miR-146a, miR-155, miR-203, miR-223	miR-124a
Systemic lupus erythematosus	miR-21, miR-61, miR-78, miR-155, miR-142-3, miR-189, miR-198, miR-298, miR-299-3p, miR-342	miR-17-5p, miR-112, miR-141, miR-146, miR-184, miR-196a, miR-383, miR-409-3p
Multiple sclerosis	miR-18b, miR-96, miR-599	
Primary biliary cirrhosis	mir-155, miR-299-5p, miR-328	miR-26a, miR-122a
Psoriasis	miR-146, miR-155, miR-203	

Table 7. MicroRNAs linked to autoimmune diseases (161, 179)

The mechanisms by which miRNAs promote autoimmunity are wide-ranging and include regional mutation of the non-coding miRNA region or target 3'-UTR, epigenetic activation, translational repression and amplification, or regulation by feedback from target protein expression. The exact causal links of the miRNAs discussed in the table have not been determined.

As discussed miR-155 is important in immune regulation and the table shows the up regulation of miR-155 in immune cells across a number of autoimmune diseases. This differential expression can also be seen with some other miRNAs, notably miR-146. MiR-146 is reportedly important in the NFkB activation pathway via the NOD2-sonic hedgehog signalling. This pathway is important for maintain gut homeostasis and development(*180, 181*).

1.2.4 Role of microRNA clinically – Therapeutics, diagnostics, prognostics?

Given the lack of understanding of the role of miRNAs in IBD it is not surprising that there is no published data on their use in the context of establishing the diagnosis or prognosis in IBD or their use therapeutically. However, there is evidence from other fields to suggest this is a promising area for future research.

MiRNAs have been studied as potential biomarkers for both diagnosis and prognosis of cancer(*182*). miR-21 may emerge as a potential biomarker for the prognosis of Colorectal cancer with implications for chemotherapy treatment and in itself may be a therapeutic target(*183*). It has been shown that a panel of 3 miRNAs, let-7f, miR-25, and miR-375 in serum, can differentiate Hepatocellular Carcinoma from healthy individuals(*184*). MiRNA profiles have been shown to be able to distinguish the underlying aetiological cause behind the development of HCC such chronic HBV, or cirrhosis. Disease staging and prognosis following treatment has also been predicted using miRNA profiling(*184*),(*185*).

There are examples of miRNAs being targeted therapeutically. Miravirsen specifically targets and inhibits miR-122 (which is only expressed in the liver) and has been shown to cause a dose dependant and prolonged reduction in HCV RNA levels in patients with genotype 1(*186, 187*). Treatment with miRNAs, miR-424 and miR-503 inhibits FGF2 and FGFR1 expression and stops the progression of Pulmonary Arterial Hypertension(*188, 189*). In multiple myeloma recent research has suggested that specifically targeting PI3K and

Ras/MAPK using synthetic miRNAs would be a potential therapy(*190*), and miR-29b is a potential target by regulating osteoclastic differentiation(*191-193*). These studies give promise that miRNAs could be targeted for therapy in other diseases including IBD.

Alicaforsen is example of an oligonucleotide therapy targeting mRNA and inhibiting the protein production in a similar way to miRNA:mRNA interaction, although it binds to the genes coding section of the mRNA rather than the 3'UTR. In this case Alicaforsen targets mRNA and inhibits the production of the ICAM-1 protein in UC and Pouchitis(*194, 195*). This reduces the its expression on the vascular endothelium, inhibiting leucocyte adherence(*196*). It also has the advantage that it is a topical treatment to be used as a nightly enema therefore specifically targeting the colon. A phase IV clinical trial has shown it to be comparable to Mesalazine enema(*195*). If suitable miRNA targets could be identified this mode of treatment may provide a way of delivering therapeutic miRNAs or anti-miRNAs to the target tissue with reduced systemic exposure.

1.2.5 Use as biomarkers in IBD

Most research into the role of miRNAs has been through analysis using microarrays and qPCR in biopsy specimens of patients undergoing colonoscopy. Studies have shown that miRNAs appear to be differentially expressed in UC compared to CD, and from active and inactive disease. This differential expression suggests that miRNAs have the potential to be used as biomarkers to confirm diagnosis, assess disease activity and perhaps to predict response to treatment in the future.

MiRNAs that could differentiate UC from CD in biopsy samples include miR-23b, mir-106, and miR-191 which are specifically raised Crohn's colitis and not in UC(*172*). This same study also identified miR-16, miR-21, miR-23a, miR-24, miR-29b, miR-126, miR-195, and let-7f, as miRNAs specifically raised in UC and not in colonic CD(*172*), whilst miR-16 and miR-21 are also raised in active terminal ileal CD(*172*). Another study has revealed that miRNAs, miR-21, miR-126 and miR-195 (known to be increased in active UC) where down regulated to levels similar to healthy controls in inactive UC patients(*161*). Also miR-125 and miR-422b, which are decreased in active UC tissue is up regulated in inactive UC(*161*).

Differential expression in the serum would make a much less invasive biomarker rather than gut mucosa; however other sources of inflammation may have to be excluded as with other serum markers of inflammation. With this in mind Wu et al showed that several serum miRNAs could distinguish IBD from other inflammatory diseases with only miR-199a-5p overlapping with SLE(*197*). This study showed that miR-28-5p, miR-103-2, miR-149, miR-151-5p, miR-340, miR-532-3p, and miR-plus-E1153 were increased in the peripheral blood samples of active UC patients and miR-505 was increased in peripheral blood of CD patients, and they could differentiate between the two diseases(*197*). Four miRNAs, miR-199a-5p, miR-362-3p, miR-532-3p, and miR-plus-E1271 were shown to be increased in active CD and not in inactive CD whereas miR-plus-F1065 is down regulated in the blood of active CD patients compared to inactive disease(*197*).

Dalal et al identified a panel of 9 miRNAs, miR-28-5p, miR-151-5p,miR-199a-5p, miR-340, miR-plusE1271, miR-3180-3p, miR-plus-E1035, miR-plus-E1153, and miR-plus-F1159 that could distinguish active UC from inactive UC, using the mayo endoscopic scoring system, by the relative up regulation of these miRNAs in the peripheral blood samples(*161*). Furthermore 5 miRNAs could distinguish active CD compared to inactive disease, using the CDAI, in the peripheral blood. miR-199a-5p, miR-362-3p, miR-532-3p, and miR-plus-E1271 are up regulated in active versus inactive disease, and miR-plus-F1065 is down regulated in active versus inactive disease(*161*). It is important to note however that these were heterogeneous groups with wide-ranging treatments. There has yet to be any research that has shown the effect of immunomodulation therapy and biologics on the miRNA profiles in these patients.

These studies suggest the role for using miRNAs in both mucosal and blood samples as biomarkers for both diagnosis and disease activity assessment.

1.3 Hypothesis and aims

1. Specific drug treatments used in inflammatory bowel disease modify the expression of Th2 cytokines mRNA and microRNA profiles in the intestinal mucosa.

I aim to assess the expression profiles of Th2 cytokines mRNA and microRNAs by qPCR in mucosal biopsies in treatment naïve patients with active UC, colonic CD, and ileal CD, and compare this to mucosal biopsies from normal healthy mucosa.

I aim to assess the expression profiles of the same Th2 cytokines mRNA and microRNAs by qPCR in mucosal biopsies in patients treated with biological and immunomodulator therapy.

I aim to use inflamed mucosal biopsies from treatment naïve patients with UC, colonic CD, and ileal CD to develop an ex vivo culture system to treat these mucosal samples ex-vivo with 5-ASA, thiopurine analogue, and antiTNF drugs, and replicate the change in Th2 cytokines mRNA and microRNA expression profiles in paired samples by qPCR.

I aim to assess the expression profiles of Th2 cytokines mRNA and microRNAs by qPCR in mucosal biopsies in patients with before and after treatment with the anti-IL13 monoclonal antibody Tralokinumab.

The rationale behind the choice of specific microRNAs and cytokines measured was based on the research already carried out by my group and other published literature. Also using targetscan specific microRNAs and cytokines were interrogated based on the cytokine function, and microRNA target gene.

miR-155 and miR-31 had been shown by our group to be involved in the targeting of IL-13 and TNFα(*198, 199*). miR-155 had also been shown to target TNFα(*174*) and SOCS1(*200*), an import Th2 and Th17 cytokine, in other research. The function of CCL18 has also be shown to be important in Th2 inflammation, particularly circulating in inflammatory bowel disease(*201*).

2. miR-31 regulates the Th2 pathway in Ulcerative colitis by directly targeting TSLP.

I aim to assess the expression of TSLP mRNA and miR-31 mucosal biopsies of patients with moderate to severe UC, and correlate the expression to show a relationship between the two.

I aim to confirm TSLP mucosal protein concentration by ELISA

I aim to demonstrate that miR-31 directly targets the 3'UTR of TSLP and inhibits its expression by cloning the 3'UTR of TSLP and performing a Dual Luciferase assay in HeLa cells

2. Materials and Methods

2.1 Equipment and Reagents

2.1.1 Software

RQ manager version 1.2	Applied Biosystems™, USA, copyright 2005
ABI PRISM® 7900HT Sequence detection system (SDS) v2.3 software	Applied Biosystems™, USA, copyright 2005
GraphPad Prism version 5.00 for Windows	GraphPad Software, California, USA
7900HT Fast Real-Time PCR System	Applied Biosystems™, USA

2.1.2 Equipment

MagNALyser	Roche Applied Science UK
DNA Engine Tetrad™ 2 thermal cycler	Bio-Rad Life Sciences, Ca, USA
Spectrophotometer, NanoDrop® ND-1000	NanoDrop Technologies Inc. DE, USA
Centrifuge 5417R	Eppendorf, UK
Centrifuge Sorvall® Legend T	Kendro Laboratory Products, Germany
Luminometer Model TD-20/20	Turner BioSystems, USA

2.1.3 Reagents

RNA Extraction

TRIzol® Reagent solution	Invitrogen, Cat # 15596018
1.4-1.6mm zirconium silicate SiLibeads	Sigmund Linder, Cat # 9315-33
Glycogen, stock concentration 20mg/mL	Roche, P/N 10901393001
Chloroform	Sigma® UK, Cat # C2432
Isopropanol	Sigma® UK, Cat # 19516
RNeasy mini kit	QIAGEN sciences, Cat # 74104
Direct-zol™ RNA Miniprep	Zymo Research, Cat # R2052

RT-qPCR

Taqman® MiRNA Reverse transcription kit	Life Technologies UK, Cat # 4366596
100mM dNTPs (with dTTP)	Life Technologies UK, Cat # 4367381
MultiScribe™ Reverse Transcriptase, 50 U/µL	Life Technologies UK, Cat # 4308228
10x Reverse Transcription Buffer	Life Technologies UK, Cat # 4319981
RNase Inhibitor, 20U/µL	Life Technologies UK, Cat # 4308224
TaqMan® Universal Master Mix	Life Technologies UK, P/N 4304437, Applied Biosystems™, USA
Random hexamers	Life Technologies UK, Cat # 58002113
RNU44	Life Technologies UK, Cat # 4427975, assay id 001094
hsa-miR-155	Life Technologies UK, Cat # 4427975, assay id 000479
hsa-miR-146a	Life Technologies UK, Cat # 4427975, assay id 002163
hsa-miR-31	Life Technologies UK, Cat # 4427975, assay id 001100
GAPDH	Life Technologies UK, Cat # 4331182
TNFα	Life Technologies UK, Cat # 4331182
CCL18	Life Technologies UK, Cat # 4331182
SOCS1	Life Technologies UK, Cat # 4331182
MicroAmp™ Optical 384 well reaction plate	Life Technologies UK, Cat # 4309849
ABI PRISM® Optical Adhesive Covers	Life Technologies UK, Cat # 4311971

Ex Vivo Tissue Culture

AQIX® 'Ready to Use' Kit Tissue Culture Media	Liquid Life UK, Cat # RS-1
5 Aminosalicylic Acid	Cambridge Bioscience Ltd, Cat # CAY70265
6-Thioguanine	Cambridge Bioscience Ltd, Cat # T2835
Adalimumab	AbbieVie
Infliximab	Merck

TSLP ELISA

BCA Protein Assay Kit	Thermo Fisher Scientific Inc, Leicestershire
Human TSLP Immunoassay kit	R&D Systems Inc, USA

Cloning and Dual luciferase system

Agarose	Thermo Fisher Scientific Inc, Leicestershire
Dual-Luciferase Reporter Assay System	Promega UK Ltd, Hampshire, UK
Opti-MEM® I Reduced-Serum Medium (1X)	GIBCO®
PBS (x10)	GIBCO®
D-MEM Media - GlutaMAX™-I	Life Technologies, Paisley, UK
Pfu DNA Polymerase 100U	Promega UK Ltd, Hampshire, UK
QIAquick Gel Extraction Kit	Qiagen, Sussex, UK
Restriction enzymes	New England Biolabs, Herts, UK
SmartLadder	Eurogentec Ltd., Hampshire, UK
TOPO® TA Cloning® Kit (with pCR®2.1-TOPO®)	Life Technologies, Paisley, UK
Plasmid Miniprep Kit	Qiagen, Sussex, UK
Plasmid Maxiprep Kit	Qiagen, Sussex, UK

2.2 Sample Collection

I recruited patients and consented them in the Endoscopy department at Southampton General Hospital, at the weekly IBD colonoscopy list. Up to 8 biopsies were taken of affected or unaffected Sigmoid colon and/or ileum from patient with Ulcerative Colitis or Crohn's Disease.

Normal mucosal biopsies were obtained from consenting adults who were having colonoscopy for polyp or colorectal cancer surveillance.

Demographic data was collected according to appendix 1. From this data CDAI, Harvey Bradshaw Index, Truelove and Witt Score, And the Mayo score for UC could be calculated.

I placed the samples directly into cryovials and they were immediately snap frozen in liquid nitrogen in the endoscopy room, or for the explant tissue culture samples, 6 biopsies were place directly into 1mL of Aqix© tissue culture media.

As well as patients being phenotyped in detail to accurately record disease activity using the CDAI, Harvey Bradshaw index and Mayo score for UC, an accurate treatment history was taken so that patients could be grouped into treatment groups. This was key, so that I could accurately determine the effects of treatment on cytokine and microRNA profiles in specific patient and treatment groups.

It is important to note that the endoscopic scoring was performed and agreed by myself and the endoscopist performing the procedure. We were not blinded to each other's score. There is potential for inter-observer variability with the scoring of these patients.

2.2.1 In vivo and ex vivo analysis of Drugs effects on microRNA and cytokine expression

I collected mucosal biopsies from patients with treatment naïve active and inactive UC and patients with treated UC on monotherapy with 5-ASA, purine analogues, or anti-TNF therapy. These biopsies were snap frozen or placed in Aqix© as described. I also collected mucosal biopsies from patients with treatment naïve active colonic Crohn's disease, treatment naïve inactive colonic Crohn's disease, and treatment naïve ileal Crohn's disease, or on monotherapy with 5-ASA, purine analogues, or anti-TNF therapy were taken. Patients were excluded if they had PSC or other undiagnosed liver abnormalities, and / or

comorbidity requiring the use of immunosuppressive or immune altering medication. Control samples were obtained from healthy individuals with a macroscopically and histologically normal intestine, undergoing colonoscopy for polyp surveillance.

2.2.2 Tralokinmab trial patients(*202*)

I was given access to RNA that had been collected from individuals aged 18-75 years, with moderately to severe active UC based on a Mayo score of greater than 6, and endoscopy sub-score greater than 2. Patients on treatment with 5-ASA, low dose corticosteroids, and purine analogues at stable doses were included, however patients with recent anti-TNF therapy or high dose corticosteroids, previous surgical resection, fulminant colitis or toxic megacolon, or an alternative diagnosis were excluded including concomitant treated colonic infection or blood born viruses, were excluded. RNA was isolated from whole mucosal biopsies these patients at baseline, and at 12 weeks following fortnightly 300mg subcutaneous Tralokinumab administration(*202*).

The exclusion criteria were: history of surgical resection or any planned bowel surgery, acute fulminant colitis or toxic megacolon, disease limited to proctitis, alternate aetiology of colitis such as ischaemic colitis, treatment with anti-TNF-α within 8 weeks of randomisation, treatment with ciclosporin, tacrolimus, methotrexate or mycophenolate mofetil (within 30 days), any live attenuated vaccine within 4 weeks, clinically significant infection (e.g. requiring antibiotics or antiviral medications) within 4 weeks of randomisation, positive baseline screening test for hepatitis B or C, tuberculosis or HIV infections or untreated systemic helminth parasitic infestation, use of antiretroviral medications, primary immunodeficiency disorder), malignancy (other than treated basal cell carcinoma or *in situ* cervical carcinoma), and active liver disease(*202*).

2.2.3 TSLP ELISA

I collected mucosal biopsies from patients with moderate to severe active UC as defined by a Mayo score of 6 or greater and endoscopic Baron score of 2-3. All patients were treatment naïve. I also collected mucosal biopsies taken from patients with inactive disease, with a Mayo score of 0-1 and Baron Score of 0. Patients were excluded if they had PSC or other undiagnosed liver abnormalities, and / or comorbidity requiring the use of immunosuppressive or immune altering medication. Control samples were obtained from

healthy individuals with a macroscopically and histologically normal intestine, undergoing colonoscopy for polyp surveillance.

2.3 Ethics Approval

South West Hampshire Research Ethics Committee granted ethics approval for eight biopsies to be taken from each consenting patient.

Reference Number 10/H0502/69

See Appendix 2.

2.4 RNA Extraction from Mucosal biopsies.

I removed the biopsies stored at -80^{o}C and thawed them on ice.

I placed individual biopsies into 1mL of TRIzol with 1000µL 1.4-1.6mm zirconium silicate SiLibeads in 2.0mL Microtubes. I then homogenised the samples in a MagNALyser for 20 seconds and at a speed of 6300rpm. After this, the sample was cooled on ice for 5 minutes and the homogenisation step was repeated until I could observe the biopsy had been completely homogenised. I made sure to cool the sample on ice after each 20 second burst of homogenisation.

I processed 300µL of the homogenate in 1.5mL reaction tubes, whilst the rest was stored at -80^{o}C for future use. I added 67µL Chloroform to each sample, vortexed for 15 seconds, and then I left the sample to settle on ice for 10 minutes. I then centrifuged the sample at 13000rpm at 4^{o}C for 40 minutes, to separate the aqueous and organic phases.

The clear aqueous phase was separated into 1.5mL reaction tubes, and I added half the supernatant's volume of isopropanol. The ratio of supernatant to isopropanol should be about 2:1, so the isopropanol volume may have to be altered. I then added 1µL glycogen (20mg/mL) to each sample. I mixed the sample by vortexing for 10 seconds and then leaving for 10 minutes at room temperature. I then precipitated the samples in the -80^{o}C freezer for at least 30 minutes. The samples were then thawed on ice, and I centrifuged the samples at 13000rpm at 4^{o}C for 30 minutes, in order to precipitate the RNA and glycogen into a visible pellet. I then removed the supernatant (aqueous phase and isopropanol) leaving the glycogen pellet taking care not to disturb the pellet. I then proceeded to the washing steps, adding 900µL 100% ice cold Ethanol to the pellet and leaving for 10 minutes at room temperature. The RNA was then pelleted again by spinning at 13000rpm, 4^{o}C for 10 minutes. I improved the quality of the RNA by repeating the washing step, by removing the ethanol and washing with 900µL 75% ethanol. I then pelleted the RNA by centrifugation again at 13000rpm, 4^{o}C for 10 minutes

I removed the supernatant, taking off as much as possible, taking care not to lose the pellet, which should still have been visible. I then performed a quick spin in the centrifuge to remove any further supernatant as much as possible. I then let any remaining ethanol and water evaporate. It was important to eliminate every trace of ethanol as this could affect

subsequent enzymatic reactions such as reverse transcription and qPCR. The samples were not left to dry for long as this could affect the resuspension in water. Once dry I added 30-50µL RNA free H_2O on ice and vortexed to redisperse the pellet. I then stored the RNA samples at -80^oC as soon as possible until they were required.

I quantified the amount of RNA in each sample, by using 2µL aliquots of each sample to analyse on the NanoDrop® Spectrophotometer at a wavelength of 260nm.

The 260nm/280nm and 260nm/230nm absorbance ratios were used to assess purity and samples used only if ratios >1.8.

2.5 RNA Extraction using the RNeasy Minikit

I removed the previously collected tissue samples that had been snap frozen from storage in the -80^{o}c freezer.

I placed each tissue sample directly into 1µL TRIzol Lysis Reagent in a MagNALyser tube for disruption and homogenisation. I then homogenised the sample immediately using the MagNALyser for 40 seconds. Once I had observed that the sample had been completely homogenised I removed 250µL of the supernatant into a new 1.5mL reaction tube. I then placed the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min. This step promoted dissociation of nucleoprotein complexes.

Then I added 50µL chloroform to the homogenate and capped the tube securely before vortexing the tube vigorously for 15 seconds. Thorough mixing was important for subsequent phase separation. I then placed the tube containing the homogenate on the benchtop at room temperature for 15 min**.** After this I separated the sample into the aqueous and organic phase by centrifugation for 15 min at 13,000 x *g,* 4°C.

I then transferred the upper aqueous phase to a new collection tube, and I added 1.5x volume (usually 225 µL) of 100% ethanol and mixed thoroughly by pipetting up and down several times. A precipitate occasionally formed after the addition of ethanol, but this did not affect the RNeasy procedure. I pipetted the sample, including any precipitate that may have formed, into an RNeasy Mini spin column that had been placed in to a 2 mL collection tube. I closed the lid gently and centrifuged at 10,000 rpm for 15 s at room temperature (15–25°C). I discarded the flow-through.

I then added 700µL buffer RWT to the RNeasy Mini spin column. Again, I closed the lid gently and centrifuged for 15 s at 10,000 rpm to wash the column. I discarded the flow-through.

I then added 500µL Buffer RPE into the RNeasy Mini spin column, closed the lid gently and centrifuged for 15 s at 10,000 rpm to wash the column again. I discarded the flow-through.

In order to improve the quality of the RNA I added another 500µL Buffer RPE to the RNeasy Mini spin column, and centrifuged for 15 s at 10,000 rpm to wash the column. I discarded the flow-through.

I then centrifuged the column at 13000rpm for 2 minutes. This long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution, as residual ethanol in the RNA sample may interfere with downstream enzymatic reactions. Following this centrifugation, I removed the RNeasy Mini spin column from the collection tube carefully, so the column did not contact the flow-through. Otherwise, carryover of ethanol could have occurred.

I then transfer the RNeasy Mini spin column to a new 1.5mL collection tube. I pipetted 50µL RNase-free water directly onto the RNeasy Mini spin column membrane. I closed the lid gently and centrifuged for 1 min at 10,000 rpm in order to elute the RNA.

I quantified the amount of RNA in each sample, using 2µL aliquots of each sample to analyse on the NanoDrop© Spectrophotometer at a wavelength of 260nm.

The 260nm/280nm and 260nm/230nm absorbance ratios were used to assess purity and samples used only if ratios >1.8.

2.6 RNA Extraction using Direct-zol™ RNA Miniprep

I removed the biopsies stored at -80°C and thawed them on ice.

I placed each biopsy sample in 1mL of TRIzol with 1000µg 1.4-1.6mm zirconium silicate SiLibeads in 2.0mL Microtubes. I then homogenised the sample in a MagNALyser for 30 seconds and at a speed of 6300rpm. I then cooled the sample on ice for 5 minutes and repeated the homogenisation step until I had observed that the biopsy had been completely homogenised, making sure to cool on ice after each 30 second burst of homogenisation. I then took half the supernatant and placed it into a new RNAse free tube. I then centrifuged the sample at 13000rpm for 1 minute to remove any particulates. I then carefully transferred the supernatant into a new RNAse free tube, leaving behind any pelleted cell particulates.

I then added an equal volume of 95-100% ethanol directly to the sample homogenate and mixed well by vortexing. I then loaded the supernatant into a Zymo-Spin™ IIC Column in a collection tube and centrifuged for 1 minute. I then transferred the column to a new collection tube and discarded the collection tube containing the flow-through. I then proceeded to the washing steps.

I added 400 µL Direct-zol™ RNA PreWash to the column and centrifuged for 1 minute, and discarded the flow-through. I repeated this step, and then added 700 µL RNA Wash Buffer to the column and centrifuged for a further 1 minute, and discarded the flow-through. To ensure complete removal of the wash buffer, I centrifuged the column for an additional 2 minutes in an emptied collection tube. I then transferred the column carefully into an RNase-free tube.

I then added 30-50µL of DNase/RNase-Free water directly to the column matrix and centrifuged at maximum speed for 1 minute to elute the RNA. The eluted RNA could then be used immediately or stored at -80°C. I Quantified the amount of RNA in each sample using 2µL aliquots of each sample to analyse on the NanoDrop© Spectrophotometer at a wavelength of 260nm.

The 260nm/280nm and 260nm/230nm absorbance ratios were used to assess purity and samples used only if ratios >1.8.

In order to perfect my and assess my RNA extraction techniques I had to do several experiments in order to improve the quality of my RNA extracts. These involved cleaning my RNA extracts and assessing quality by RNA electrophoresis as described in section 2.7 and 2.8. I had to change my RNA extraction techniques several times over many months, and the published methods here are the final versions.

2.7 RNA Acid Phenol Extraction and Clean Up

I created a SDS 10x stock solution by adding 1 volume 500mM EDTA, and 1 volume Tris HCl 1M pH 7.5 together.

I started with a set volume of RNA solution. I then add 1/9 volume of SDS 10% Buffer and mixed by vortexing for 10 seconds. I then added 1/9 Volume NaAc 0.3M pH 5.2 and twice the sample volume of Phenol:Chloroform:Isomyl Alcohol solution (sigma-aldrich). I then mixed the sample thoroughly by vortexing for 1 minute. I then separated the organic and aqueous phases by centrifugation at maximum speed for 2 minutes. I removed the top aqueous phase and added to it, twice the phase volume of Chloroform before vortexing for 1 minute. I then centrifuged at maximum speed for 2 minutes.

I then again removed aqueous phase and added 2.5 times the phase volume of ice cold 100% ethanol and 5µL Glycogen. I ensured this was mixed by vortexing for 1 minute. I then precipitated the RNA by placing the sample at -80^{o}c for 1 hour.

I then pelleted the RNA and glycogen by centrifugation at maximum speed for 10 minutes.

I then washed the pellet three times in 750µL ice cold 75% ethanol leaving on ice for 5 minutes and repelleting by centrifugation between each wash. I then air dried the pellet until it was translucent and added 30µL RNA free water to resuspend the RNA. The sample could then have been frozen at -80^{o}c or used for continuing on to reverse transcription or RNS electrophoresis.

I Quantified the amount of RNA in each sample by using 2µL aliquots of each sample to analyse on the NanoDrop© Spectrophotometer at a wavelength of 260nm.

The 260nm/280nm and 260nm/230nm absorbance ratios were used to assess purity and samples used only if ratios >1.8.

2.8 RNA electrophoresis

I performed RNA electrophoresis under denaturing conditions in 2.2M formaldehyde according to Maniatis et al using the MOPS buffer system(*203*). RNA under these conditions is fully denatured and migrates according to the $\log_{10}$ of its molecular weight.

I created a 10 x MOPS buffer using equal volumes of 0.2M morpholinopropanesulphonic acid, 50mM sodium acetate, and 5mM EDTA. I adjusted the pH of the buffer to pH 7.0 by adding 1M NaOH. I then bottled the buffer and sterilised it by autoclaving.

I then created an RNA denaturing buffer in a fume hood by mixing 10mL 100% deionized formamide, 3.5mL 40% formaldehyde, and 1.5mL 10 x MOPS buffer (as described). Formamide was deionized by stirring 100mL with approximately 20g of Amberlite MB3 (or MB1) ion exchange resin for 15 minutes.

I prepared a 1.5% Agarose gel by melting 0.75g of agarose in 37mL distilled water, cooling to approximately 60°C so the flask could be handled and adding 8.75mL 40% formaldehyde and 5mL 10 x MOPS to give 2.2M formaldehyde and 1 x MOPS, respectively. This gave a total volume of 50mL.

I created a solution of 1 x MOPS, and 2.2M formaldehyde, to use as a buffer to perform the electrophoresis in.

I prepared the RNA samples by adding up to 25mg of RNA in a maximum of 5µL sterile H_2O, to 15µL RNA denaturation buffer. I added 1 µL 10mg/mL ethidium bromide to aid visualisation of RNA after electrophoresis. Immediately prior to loading, I heated the RNA samples to 65°C for approximately 10 minutes to denature any secondary structure. I then cooled the sample on ice for 2 minutes and 2µL of sterile loading buffer was added.

I then loaded the samples onto the gel and electrophoresis was performed at no more than 5V/cm, with occasional buffer recirculation, until the leading bromophenol blue dye front has migrated approximately three quarters of the length of the gel. Visualisation of RNA was achieved by irradiation with short wave (254nm) UV light. Typical markers of RNA quality were regarded as 18S (~1900bases) and 28S (~4800bases) RNA molecules.

2.9 Explanation of Laboratory techniques

As you can see I used multiple techniques for both RNA extraction and qualification.

I spent many months trying to perfect the RNA extraction technique for the different experiments the tissue involved was going to be used for.

With samples that were snap frozen the manual RNA extraction technique with TRIzol offered the best yield of DNA, however, this technique needed alteration at several stages to both improved the quantity and quality of the RNA extracted.

Firstly, the homogenisation stage required careful manipulation in order to reduce RNA breakdown. Shorter bursts of homogenisation with cooling on ice gave better results.

Secondly reducing the amount of glycogen used to the minimal amount in order to visual the pellet improved the quality of the RNA using the spectrophotometer reading and when assessing the RNA quality using RNA electrophoresis.

Thirdly, it was very important to make sure that the alcohol used to clean the RNA pellet was thoroughly removed before dispersing the RNA in RNAse free water. This was not only done by serial centrifugation and pipetting out of the alcohol, but also by air drying the RNA pellets before dispersing them in water.

The RNA extraction from the tissue samples used in the cell culture model did not seem to be as effective using the manual method using TRIzol. I therefore conducted experiments to compare both the quantity and quality of RNA extracted from these samples using the two column techniques described. The Directzol kit provided the best quantity, as measured on the spectrophotometer and the best quality when analysed using RNA electrophoresis.

2.10 MicroRNA Reverse Transcription and Real Time qPCR

2.10.1 MicroRNA Reverse transcription

The RNA samples were diluted to 10ng/µL by taking 1µL of RNA sample at a known concentration and I added it to the appropriate volume of RNA free water as shown:

Volume of RNAse free water = Sample RNA (Concentration (ng/µL)/10) - 1 in µL.

I then mixed each sample by vortexing and centrifuged at maximum speed.

I created a separate stock Mastermix for each microRNA or housekeeper gene using table 8, and then vortexed and centrifuged to maximum speed:

Reagent	Volume per sample (µL)
RNA free Water	4.081
10x Reverse Transcription Buffer	0.75
100mM dNTPs	0.075
Reverse transcriptase	0.5
RNAse inhibitor	0.094
Specific MicroRNA/Housekeeper RT Primer	1.5

Table 8. Reagents for MicroRNA reverse transcription

I combined 1µL of diluted RNA to 7µL of Mastermix in separate reaction tubes for each microRNA or Housekeeper gene being investigated.

I then ran the reverse transcription reaction on the Tetrad™ 2 thermal cycler for 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and then 14°C until transferred to the -20°C freezer for storage.

2.10.2 MicroRNA Real Time qPCR

I created Mastermixes for each primer as per table 10 (see table 9 for primers used).

Using a MicroAmp™ Optical 384 well reaction plate, I added 1.0µL of cDNA and 4.0µL of mastermix to each well. Each sample was performed in duplicate for each microRNA of interest or housekeeper gene.

House keeper gene
RNU44
MicroRNA of Interest
miR-155
miR-146
miR-31

Table 9. TaqMan microRNA Probes used for microRNA RTqPCR

Reagent	Volume per sample x 2
Taqman Universal PCR Mastermix buffer	2.5µL
RNA free H_2O	1.25µL
PCR Primer (table 8)	0.25µL

Table 10. Recipe for microRNA RTqPCR

In order to ensure the samples were mixed and at the bottom of the well I centrifuged the plate at 2000rpm for 2 minutes and then the PCR reaction was run on the 7900HT Fast Real-Time PCR System using the following thermal cycling conditions; stage 1, 2 minutes at 50°C followed by 10 minutes at 95°C. Stage 2, 15 seconds at 95°C followed by 1 minute at 60°C. Stage 2 is repeated for 40 cycles.

2.11 mRNA Reverse Transcription and Real Time qPCR

2.11.1 mRNA Reverse Transcription

Following Quantification of the RNA in each sample, there was further dilution as follows depending on the initial RNA concentration of the sample. I did this in order to dilute the RNA top concentrations that made is easier to do the reverse transcription reaction

Sample concentration	volume H_20 added (µL)	resultant concentration (ng/µL)
0-500 ng/µL	0	NanoDrop© reading
500-1000 ng/µL	50	reading/2
1000-2000 ng/µL	100	reading/3
Over 2000 ng/µL	150	reading/4

Table 11. RNA dilution calculations

I then created a stock mastermix for the reverse transcription reaction by mixing 1.0µL Reverse Transcription Buffer (10x), 0.4µL dNTPs (100mM, with dTTP), 1.0µL random hexamers, 0.5µL RNase Inhibitor (20U/µL) and 0.5µL MultiScribe™ Reverse Transcriptase (50 U/µL). The volumes indicated were per sample being analysed. Either 100, 200, or 400ng RNA was calculated per sample and I made these up to 6.6µL. I then added to 3.4µL of mastermix to each sample. I made sure this was all done on ice, to avoid the RNA denaturing.

I then ran the RT reaction on the Tetrad™ 2 thermal cycler at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and then kept at 20°C until I transferred the samples of cDNA to the -20°C freezer for storage.

2.11.2 mRNA Real Time qPCR

I diluted cDNA samples to a ratio 1:10 using RNA free water.

I then created a mastermix for each primer as per table 13 (see table 12 for primers used) Then, using a MicroAmp™ Optical 384 well reaction plate, I added 1.25µL of diluted cDNA and 3.75µL of mastermix to each well. Each sample was performed in duplicate for each gene of interest or housekeeper gene.

House keeper gene
GAPDH
Gene of Interest
SOCS1
TNFα
CCL18

Table 12. TaqMan mRNA Probes used for RTqPCR

Reagent	Volume per sample x 2
Taqman Universal PCR Mastermix buffer	2.5µL
RNA free H_2O	1.0µL
PCR Primer (table 11)	0.25µL

Table 13. Recipe for RTqPCR

In order to ensure the samples were mixed and at the bottom of the well I centrifuged the plate at 2000rpm for 2 minutes and then the PCR reaction was run on the 7900HT Fast Real-Time PCR System using the following thermal cycling conditions; stage 1, 2 minutes at 50°C followed by 10 minutes at 95°C. Stage 2, 15 seconds at 95°C followed by 1 minute at 60°C. Stage 2 is repeated for 40 cycles.

2.12 Explant Cultures and Drug treatments

As described in section 2.2.1, I collected 6 biopsies from the mucosa of either treatment naïve colonic sigmoid CD, Ileal CD, sigmoid UC, or normal control patients and placed in 1mL of Aqix© cell culture medium. Once back in the lab I separated the biopsies into 6 separate wells of a 96 well U-bottom cell culture plate with 200µL Aqix© cell culture medium in each well. I then treated the biopsies with drugs as per table 14 by adding the drug to the Aqix© solution and biopsy in each well.

Well	Drug	Drug concentration in solution	In vitro concentration in 200µL Aqix©
1	Untreated Control	Not applicable	Not applicable
2	2µL 5-ASA solution	40µg/mL	400ng/mL
3	2µL 6-Thioguanine solution	500µg/mL	5µg/mL
4	2µL Infliximab solution	5mg/mL	50µg/mL
5	2µL Adalimumab solution	5mg/mL	50µg/mL
6	2µL DMSO, as a control for 5-ASA	Not applicable	Not applicable

Table 14. Showing the drug concentration used in the ex vivo experiment. Drug concentrations were used from previous in vitro work(204, 205), or as per manufacturers guidelines with 6-Thioguanine.

The biopsies were then incubated for 24 hours at 37°C, with a plate cover. I then removed the treated biopsies from the wells and placed each one to a separate 2.0mL MagNALyser© Microtube with 1000µL of TRIzol with 1000µg 1.4-1.6mm zirconium silicate SiLibeads. I then homogenised the samples in the MagNALyser© for 20 seconds cycles and at a speed of 6300rpm with 5 minutes cooling on ice between cycles. The samples were then stored at -80oC or I proceeded to RNA isolation using the Direct-zol™ RNA miniprep method in section 2.6.

2.13 Enzyme Linked ImmunoSorbant Assay

The colonic biopsies that I had snap frozen in liquid nitrogen and stored at -80^{o}C were thawed on ice. The samples were homogenised by four cycles of freeze thawing with liquid nitrogen in 200µL PBS and 8 µL protease inhibitor. The samples were vortexed between each cycle.

I then removed the insoluble material by centrifugation at 13200rpm in order to pellet the cell debris and the protein rich supernatant was aspirated for analysis.

I used a Quantikine Human TSLP Immunoassay from R&D Systems to analyse TLSP protein concentrations in tissue. The reagents were prepared as per the kit protocol after being brought to room temperature.

I reconstituted the wash buffer concentrate (25-fold concentrated solution of buffered surfactant with preservatives) by adding 20mL of concentrate to 480mL of distilled water.

The TSLP Standard (recombinant human TSLP in a buffered protein solution with preservatives; lyophilized) was reconstituted with 1.0mL of distilled water to produce a stock solution of 20000pg/mL and I gently agitated this for 15minutes. Using 9 polypropylene tubes, I added 900µL of Calibrator Diluent RD6-10 (buffered protein solution with preservatives) into tube 1. I then added 500µL of Calibrator Diluent RD6-10 into the remaining tubes. Using the 20000pg/mL stock solution I had already made a dilution series was produced. 100µL was added to the first tube. Then I added 500µL of the standard from tube 1 to tube 2 and so on to tube 9. Each tube was mixed thoroughly before the next transfer. The concentrations I had reconstituted were 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.8125pg/mL respectively. The Calibrator Diluent served as the zero standard (0pg/mL).

Any excess microplate strips (96 well polystyrene microplate coated with a polyclonal antibody against TSLP) I removed from the plate frame. I added 100µL assay diluent RDX1 to each well of the precoated microplate. Then I added 50µL of each concentration of TSLP standard or sample supernatant per well.

The plate was then covered and incubated at room temperature for 2 hours.

I then aspirated each well and washed each well 4 times with 300µL of wash buffer. Complete removal of wash buffer was ensured after each wash by inverting the wells against blotting paper.

Then, I added 200µL of TSLP Conjugate (polyclonal antibody against TSLP conjugated to horseradish peroxidase with preservatives) to each well and I recovered the plate and incubated the samples for a further 2 hours at room temperature. I then repeated the 4-cycle wash step.

Colour reagent A (stabilized hydrogen peroxide) and Colour reagent B (stabilized chromogen (tetramethylbenzidine) were mixed together in equal volumes within 15 minutes of use to create a substrate solution. I added 200µL of this Substrate Solution to each well, and I incubated the plate for 30 minutes at room temperature, making sure the plate was protected from light, by placing in to a drawer.

50µL Stop solution (2 N sulfuric acid) was then added to each well. I gently agitated the plate to ensure mixing was performed. Within 30 minutes the optical density was analysed using a microplate set to 450nm with wavelength correction set to 540nm.

I plotted the standard optical density against the standard TSLP concentration. I created a regression curve and equation using Microsoft excel software. The sample TSLP concentrations could then be determined using the corrected optical density for each sample.

2.14 BCA Protein Assay

In order to correct for biopsy volume when analysis of the TSLP ELISA protein concentration was performed, I performed a BCA protein assay on each sample.

The same supernatant used for each samples TSLP protein ELISA was used to assess total protein concentration.

I used the Pierce® BCA Protein Assay Kit in order to achieve this.

The Albumin standard was created using a stock solution of 2000µL/mL and PBS as a diluent using the table below (table 15)

Vial	Volume of Diluent(µL)	Volume (µL) and Source of BCA	Final BCA Concentration(µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	100
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0

Table 15 Preparation of Diluted Albumin (BCA) Standards

Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium Hydroxide) and reagent B (4% cupric sulphate) were mixed together at a ratio of 50:1 to create the working reagent. I used 10mL reagent A, and mixed it with 200µL reagent B.

In triplicate, I added 5µL of standard or sample to each well with 100µL of the reconstituted reagent. I then covered the microplate and incubated it at 37^{o}C for 30 minutes. The microplate was then cooled to room temperature and I measured the optical density at 560nm in a microplate reader.

The total protein concentration for each sample was then used to correct for the TSLP protein concentration in each sample.

2.15 Cloning

2.15.1 Primer design

I designed the primers for cloning the 3'UTR_TSLP using the blast primer design website (Figure 4 and Figure 5) using a template of the entire sequence of the 3'UTR_TSLP as seen in Figure 3. The target site for miR-31 was identified using the bioinformatics website targetscan and then I designed primers with mutation of the site with the XbAI enzyme (Figure 6 and Figure 7). These primers were ordered from Invitrogen Life Biosciences.

5':ACCATCTTTATTATGGTCATATTTCACAGCACCAAAATAAATCATCTTTATTAAGTAGATGAAACATTAACTCTAACTGTG
ACAAAGAAGACCACAAATAGTTATCTTTTAATTACAGAAGAGTTTCTTAACTTACTTTTGTAAGTTTTTATTGTGTAAGTTTA
TAATGCAGGGGAAGTACTACTCCTCAAATGTTGAGGGAAGCTTCCATAACATTGATGACTGGCTTCATGGCAGTAATTCTC
GGCTGTAGTTGCATAAGCATTGCTCAAGAGGAAAATCCAAAAGTGCAGCAGGAGAACTCTTTTCCCTGAAAAAGGAAAAA
TATTGAACTCAATGATAGCACCTAAACTTACATTTAAAAGACAGACATTCCTTCTACATGTAATGACACTTCTTGTGTTAAAC
TAAAAATTTACAAGAGAAGAAAGTGAAAGCAAATGGGGTTTCACAAATAGTTGTAAATATAGTGAAGCAATTTGAAATAA
TTTTCAAGCAAAGTATTGTGAAAGTATTCTAAGCCAAGTTTTAAATATTATCTAACAGACAAGAGTGGTATATACAAGTAG
ATCCTGAGAAGTACCTTTGTTACAGCTACTATAAATATACATATAAATTATAGAATCTACTTTAATTTATTTTGTGAACACTT
TTGAAAATGTACATGTTCCTTTGTAATTGACACTATATATTTCTTAATAAAATAATTCTCAAATTTGTTTCTTATGAATCATCT
CTCAAATCTAGTTAGACAATTTGCACACATACTTTTCTAAGGGACATTATCTTCCTTCAGGTTTTTACCTCCACTCATCCTTAG
AGCCCACTGACTGCTCCCCTTTATACCTGTTGGCCCTGCCTATAGGAGAGAATATTTGGAGATAGGCAGCTTCAGGATGCA
TTGCAATCATCCTTTTCTTAAATTATGTCACTAGTCTTTTATTTTTTCCCCTCTTGAACTTTCCTCACACCTGGAAGAAACAAA
GTAGGAAAAAGTGAACAGGGGATGTCAAATCGATTCTTGAATTCCCGCTGCAAGCTAGAGCCGCAGGCACCCTCTCACTC
AATTTCCACTCAGAACCCTATAAACACCAGTGGGAAGGGCAACCCACTGCACGTGGGAATGCACTGATTTTTCCTAGGAGT
AGACATGTTCCTCTAATTACTCCCTGAGGGTTAGTTGGGGCTAAACCATGACAGAAGTGGGGAAGTTCAATGTCCTTAAAT
CCATCTTACTTGCCAACAGGTAAGAGGAAGCTTACATTACATGTCCAGTCCACATTTAAAGAGCACTTACTGTGGAACAAG
CCTTCAGCCAAACAATGGGGATAGAAAAGTAGGTAAGACTCAGCCTTTGTCCAGAGAAGCTCAGGGTATAGCTGAATAG
GCAGTTTCTTTTGTCCTGAGGAAAATCAGGACATGCCTGCTTTCTAAAAATCTTCCTCTGAAGACCTGACCCAAGCTCTTAA
ATGCTATTGTAAGAGAAATTTCTTTGTCTATTAACTCCATTTTAGTAGGGATTCACTGACTAGATTTTACTGAACTATGAAA
ATAAATACACATAATTTTTCACAAAATTTTGGGCCCAATTCCCCTAAAAGAATTGAGGATTAGGGAGAAAGGAGACAACTC
AAAGTCATCCCATTAAGTGCAGTTTCTTTGAATCTTCTGCTTTATCTTTAAAAATTTGTATAATTTATATATTTTATTCTATGT
GTTCCATAGATATCTTAATGTAAAATTAGTCATTTAAATTACACTGTCAATTAAAAGTAATGGGCAAGAGATTGCATCATAC
TAATTTAGTAAGAACGTTCCCAAATGTTGTAACAATGTGGATCATACATCTCTGGTTTTTTAAATGTATTGAGGCTTTCTTG
GTGGACTAGTATAGTATACGGTCAGTTATGTCAATGTTTCATGGTCAATAAAAAGGAAGTTGCAAATTG:3'

Figure 3. 3'UTR_TSLP sequence with the miR-31 target identified by bioinformatics highlighted in yellow and the primer targets identified highlighted in pink

FOR_XbAI_3'UTR_TSLP

5' TCTAGA AGGCACCCTCTCACTCAATT 3'

Figure 4. Forward XbAI_3'UTR_TSLP primer sequence

REV_ Not1_3'UTR_TSLP

3' GCGGCCGC GGCATGTCCTGATTTTCCTCA 5'

Figure 5. Reverse Not1_3'UTR_TSLP primer sequence

FOR_MUT_3’UTR_TSLP

5’ GTCCTTAAATCCATCTTATCTAGAAACAGGTAAGAGGAAG 3’

Figure 6. Forward mutated miR-31 target 3'UTR_TSLP primer sequence, miR-31 mutated target site highlighted in yellow

REV_MUT_3’UTR_TSLP

3' GGAATTTAGGTAGAATAGATCTTTGTCCATTCTCCTTCGA 5'

Figure 7. Reverse mutated miR-31 target 3'UTR_TSLP primer sequence, miR-31 mutated target site highlighted in yellow

2.15.2 Amplification of the 3’UTR_TSLP

I created two mastermixes, as per table 16, in order to perform a PCR for the 3’UTR of TSLP at different temperatures. This was done to assess the optimum temperature by which the best PCR result could be achieved.

	Volume
Forward Primer (1µg/µL)	0.2µL
Reverse Primer (1µg/µL)	0.2µL
GoTaq Flexi Buffer	4µL
dNTPs	0.4µL
RNAse Free Water	12.1µL
Whole DNA (diluted to 7ng/µL)	1µL
GoTaq PCR Enzyme	0.1µL
Magnesium Chloride	2µL

Table 16. Table showing mastermix for PCR of 3’UTR_TSLP

This totalled a volume of 20µL for each mastermix. I then performed an amplification PCR reaction in the Tetrad™ 2 thermal cycler at the following program:

95°c	2 minutes	1 cycle
95°c	30 seconds	40 cycles at these temperatures
55°c or 58°c	30 seconds	
72°c	1 minute	
72°c	5 minutes	1 cycle
15°c	Forever	Continuous

Table 17. Programmed cycle on Tetrad™ Thermocycler for PCR of 3'UTR_TSLP

I then created a 1% Agarose gel using 0.5g of Agarose powder and 50mL of x1 TBE and 2.5µL Nucleic acid stain solution. The above PCR products were run on the electrophoresis to see which temperature gave the strongest band. In this case 58^{o}c was the optimum temperature for the PCR.

2.15.3 TOPO Cloning

I used a TOPO plasmid to clone the 3'UTR_TSLP using a TOPO cloning kit.

I created a 6µL TOPO cloning reaction by adding 4µL of PCR product, 1µL Salt solution, and 1µL TOPO vector to a 1.5mL reaction tube. I mixed the reaction gently at room temperature for 5 minutes on an agitator and then I placed the tube on ice. Meanwhile I slowly thawed a vial of oneshot *E-Coli* cells on ice. I was careful not to handle the bottom of the vial so not to warm the cells too quickly.

I then added 2µL of the TOPO cloning reaction to the thawed *E-Coli* cells and I incubated the cells on ice for 30 minutes. Then I heat shocked the cells for 30 seconds at 42^{o}c, without shaking in an incubator and then I placed them back on ice for 2 minutes.

Then I added 250µL of SOC Media to the cells, and the vial was capped and agitated at 37^{o}c for 60 minutes in order to mix the cells and SOC media.

The media was then spread on to a warmed LB Agar plate that had been treated with 50-100µg Ampicillin, and I incubated the plated cells at 37^{o}c overnight.

At this stage I could either pick the resultant colonies and perform a miniprep (section 2.14.4), or the plate could be stored in the refrigerator.

2.15.4 QIAgen Quickspin Miniprep Plasmid isolation

I picked separate colonies from the TOPO cloning and cultured them overnight in 5mL of LB culture media with Ampicillin, in 20mL tubes, at 37^{o}c.

I then removed approximately 3mL from each overnight culture and I centrifuged the culture product at 13000rpm in 1.5mL Eppendorf tubes in order to pellet the bacteria. The media was discarded, and I resuspended the bacterial pellets in 250µL of P1 buffer solution by vortexing thoroughly.

Then I added 250µL of P2 buffer solution from the kit to each tube and mixed by inverting the tubes 6 times, not by vortexing. I then left the tubes for 5 minutes at room temperature for cell lysis to occur.

Then I added 350µL of N3 Buffer solution was and immediately mixed by inverting the tubes 6 times, before I transferred the tubes to the centrifuge and I spun them for 10 minutes at 13000rpm.

The supernatant was drawn off and I placed it in the provided columns. The I centrifuged for 60 seconds in order to draw the supernatant through the columns into the provided collecting tubes. This flow through was discarded and I placed 750µL of wash buffer PE into the column and centrifuged through for a further 60 seconds. This flow through was again discarded and the column I centrifuged the column for a further 60 seconds to remove any residual wash buffer.

The columns were then transferred to 1.5mL Eppendorf tubes and I placed 50µL RNA grade water directly in to the centre of the column and left for 60 seconds to elute as much plasmid as possible. I then centrifuged the eluted plasmid through the column for 60 seconds, and collected it in the Eppendorf tube. The plasmid was then transferred for storage at -20^{o}c.

2.15.5 ECO Digest of TOPO_3'UTR_TSLP

For each clone I made a mixture containing the ingredients from table 18.

	Volume
10x Buffer	2µL
Plasmid (DNA)	0.5µL
ECO Enzyme	4µL
RNAse free Water	13.5µL

Table 18. Ingredients for ECO Digest of TOPO_3'UTR_TSLP

I mixed these thoroughly by vortexing and spinning down in the centrifuge for a few seconds. I then incubated the tubes at 37^{o}c for 2 hours. RNA electrophoresis was then performed using a 1% Agarose gel to determine which clones had successfully inserted the

3’UTR_TSLP in to the plasmid. These clones were sent for full sequencing and those with no mutations in the 3’UTR_TSLP were stored at -20^{o}c, all other samples were discarded.

2.15.6 pRLTK_ 3’UTR_TSLP isolation and digest

I chose one of the positive clones to isolate 3’UTR_TSLP insert. A pRLTK plasmid with TLR7 insert that had been created previously was used to isolate the pRLTK plasmid (Rupani).

I performed the digestion reaction for the 3’UTR_TSLP and pRLTK plasmid in 2 separate tubes as shown in table 19.

I incubated the tubes at 37^{o}c for at least 3 hours. The whole volume of the digestion products from each tube were then run on a 1% Agarose gel using electrophoresis to separate the inserts from the plasmids. Using a clean scalpel, I cut the 3’UTR_TSLP insert out of the gel and placed into 1 tube and I cut the pRLTK plasmid out of the gel and placed in another tub

Tube 1

	Volume
TOPO_3’UTR_TSLP DNA	20µL
Buffer 3.1	3µL
Enzyme XbaI	1µL
Enzyme Not1	1µL
RNAse Free Water	5µL

Tube 2

	Volume
pRLTK_TLR7 DNA	15µL
Buffer 3.1	2µL
Enzyme XbaI	1µL
Enzyme Not1	1µL
RNAse Free Water	1µL

Table 19. Tables showing the ingredients for digest of pRLTK_3'UTR_TSLP

2.15.7 QIAgen Gel Extraction

I isolated the pRLTK plasmid and 3'UTR TSLP from the gel by using the QIAgen gel extraction kit. The extracted gel bands were placed in two separate Eppendorf tubes as described. I then added 300µL of Buffer QG to each tube and the agitated at 50^{o}c for 10 minutes in a thermal shaker or until the agar had dissolved. I then added 1 volume (300µL) of isopropanol and I mixed each solution by vortexing. I then transferred the solutions to two QIAquick columns and I centrifuged them for 1 minute. I discarded the flow to leave the columns. I then washed the DNA in the columns with 750µL buffer PE by centrifuging through for 1 minute. After this, I discarded the flow through and a further 1 minute of centrifugation was performed to remove any residual wash buffer. I then transferred the column to a new 1.5mL Eppendorf tube. I then added 50µL DNAse free water that was warmed to 65^{o}c added in to the centre of the column to elute the DNA. I then collected the eluted DNA in to the tube by centrifuging the column for 1 minute.

The concentrations of DNA were quantified on the NanoDrop© Spectrophotometer at a wavelength of 260nm, using 2µL aliquots. If the concentration of pRLTK plasmid was below 20ng/µL or 3'UTR_TSLP insert below 5ng/µL then I concentrated the samples in a speed vacuum centrifuge.

2.15.8 pRLTK 3'UTR_TSLP Ligation reaction

The ligation reaction took place in a volume of 10µL. The ratio I used of pRLTK vector:3'UTR_TSLP insert was 2:1, and the optimum pRLTK vector amount was 50ng. I used 1µL T4 Ligase Enzyme, and 1µL T4 Ligase buffer, and I added this to 50ng pRLTK plasmid and 25ng 3'UTR_TSLP insert. I made the total volume up to 10µL with DNAse free water in a PCR tube. I incubated the reaction at 16oc overnight for ligation to occur.

I performed transformation of the pRLTK_3'UTR_TSLP plasmid ligation product into oneshot *E-Coli* cells by adding 5µL of ligation reaction product to the cells and incubating on ice for 30 minutes. Then the cells were then heat shocked for 30 seconds at 42^{o}c, without shaking, and then I placed the cells back on ice for 2 minutes.

Then I added 250µL of SOC Media to the cells, and the vial was capped and agitated at 37^{o}c for 60 minutes, in a thermal shaker.

I then spread the cells in the SOC media on to a pre-warmed LB Agar plate that had been treated with 50-100µg Ampicillin, and incubated the plate at 37^{o}c overnight.

At this stage I could either pick the resultant colonies to perform a miniprep (section 2.14.4) or the plate can be stored in the refrigerator.

Colonies were then picked and cultured overnight as described in section 2.14.3 and a QIAgen quickspin miniprep performed as described in section 2.14.4. A digest of the isolated plasmid was performed in 20µL using 4µL of plasmid DNA, and 2µL of Buffer 3.1 as described in section 2.14.6. I performed DNA electrophoresis using a 1% agar gel to determine which of the colonies picked had the 3'UTR_TSLP inserted successfully. I could then use these cultured colonies to perform QIAgen Maxipreps (section 2.14.9), to maximise the yield of the plasmid.

2.15.9 QIAgen Maxiprep

I placed 20µL of the overnight culture of cloned pRLTK_3'UTR_TSLP into a large 3L conical flask with 0.5L of fresh LB culture media with Ampicillin. This was cultured overnight at 37^{o}c. I then harvested the overnight culture by centrifuging at 600g for 15 minutes. The bacterial pellet was then resuspended in 10mL of buffer P1. I then added 10mL of buffer P2 with Lyseblue and I mixed it thoroughly by inverting 6 times so that the solution turned blue. Then I added 10mL of prechilled buffer P3 and I mixed it further by inverting 6 times, and then I incubated the solution on ice for 20 minutes, until the solution turned white or colourless. The solution was then centrifuged at 18000g for 10 minutes at 4^{o}c to separate off the clear supernatant. I would proceed to further centrifugation if the supernatant was not clear.

Meanwhile I equilibrated a QIAgen tip 500 by applying 10mL buffer QBT to the column and I allowed it to empty through the column by gravity flow into an empty flask. The clear supernatant was the applied in to the column and I allowed it to flow through by gravity flow in to a collecting flask. I then washed the column by application of 30mL of wash buffer QC, and again I allowed it to flow through by gravity flow. The DNA was then eluted with 15mL buffer QF into a clean 50mL vessel. I then precipitated the DNA by adding 10.5mL isopropanol to the eluted DNA. The solution was mixed thoroughly, and I centrifuged the solution at 15000g for 30 minutes at 4^{o}c, in order to pellet the DNA. I then carefully

decanted the supernatant leaving the DNA pellet at the bottom of the vessel. I then washed the pellet with 5mL 70% ethanol and I centrifuged at 15000g for 10 minutes, to repellet the DNA. The ethanol was decanted, and I left the pellet to air dry for 5-10 minutes.

Finally, the DNA was resuspended in 2-3mL of DNAse free water and transferred to 2 sterile Eppendorf tubes. I quantified the concentrations of DNA on the NanoDrop© Spectrophotometer at a wavelength of 260nm, using 2µL aliquots, and then I stored the DNA at -20^{o}c.

2.15.10 Site targeted Mutagenesis

Mutagenesis of the miR-31 target on the 3'UTR_TSLP was performed by using the pRLTK_3'UTR_TSLP plasmid as a template. The target site was mutated with the sequence for the XBaI enzyme target (Figures 6 and 7).

The forward and reverse mutagenesis primers from section (Figures 6 and 7 section 2.14.1) were dissolved in DNAse free water to a concentration of 1µL/µL. I then added 1µL of each primer to a sterile PCR tube with 9µL DNAse free water, so that a solution with 100ng/µL of each primer was made.

I then made the concentration of pRLTK_3'UTR_TSLP plasmid to 1ng/µL by taking 1µL of the maxiprep product, and adding the appropriate volume of DNAse free water.

I created a PCR reaction solution by using the volumes in table 20, and mixing them thoroughly. This made a final volume of 50µL. I then ran the PCR reaction in the thermocycler according to the program in table 21.

	Volume
Mutant Primers (100ng/µL)	1.25µL (125ng each primer)
Plasmid DNA (1ng/µL)	10µL (10ng)
Magnesium Chloride	3µL
dNTPs	1µL
PFU Enzyme	1µL
GoTaq Buffer	10µL
DNAse free Water	23.75µL

Table 20. Ingredients for generation of site specific mutation pRLTK_3'UTR_TSLP

95°c	5 minutes	1 cycle
95°c	50 seconds	18 cycles at these temperatures
58°c	50 seconds	
68°c	5 minutes (1min/Kba)	
68°c	7 minutes	1 cycle

Table 21. Program for PCR of site specific mutation pRLTK_3'UTR_TSLP

The resultant DNA was plasmid containing the 3'UTR_TSLP with a mutation at the binding site of miR-31. This PCR product was then stored at -20°c.

Prior to a transformation of the mutated plasmid into oneshot *E-coli* cells the non-mutated bacterial plasmid was digested from the solution by adding 1µL DNP1 enzyme and incubating at 37°c for 1 hour. I then proceeded to transformation in to oneshot *E-coli* cells using the above PCR product as described in section 2.14.8 I then plated the bacteria LB agar gel with Ampicillin and I cultured the bacteria overnight. I then picked the resultant colonies, and I cultured the colonies in LB media overnight. I then performed a QIAgen miniprep on the cultured colonies, as described in section 2.14.4

I then performed a digest of the isolated plasmid. By using 7µL of plasmid DNA, and 2µL of Buffer 3.1, I created a 20µL solution. I followed the digestion method as described in section 2.14.6 but only using the enzyme XbaI to cut the plasmid. Following this I performed an electrophoresis on a 1% agar gel to determine successful clones. A QIAgen maxiprep for the successful clone of the mutant 3'UTR_TSLP was then performed (section 2.14.9).

2.16 Transfections and Dual Luciferase system

The constructs pRLTK(-) empty vector, pRLTK_WT_3´UTR_TSLP (wild type), or pRLTK_MUT_3´UTR_TSLP (mutant site for miR-31 binding) were co-transfected with pCDNA3.1_miR-31 or pCDNA3.1(-) empty vectors into HeLa cells. I achieved normalisation by co-transfecting the cells with pGL3. Assays were measured with the Dual-Glo kit. I performed all the experiments in duplicate.

Firstly, I created a mastermix of 750µL Optimum, and 300ng pGL3 in a single Eppendorf, and then I split the mastermix equally between 3 clean Eppendorf tubes after thorough mixing. To the first tube I added 600ng pRLTK(-) empty vector, 600ng pRLTK_WT_3´UTR_TSLP was added to the second tube, and 600ng pRLTK_MUT_3´UTR_TSLP to the third tube. Then I split each tube equally into 2 long glass reaction tubes. In to tubes 1, 3, and 5, I added 2µg pCDNA3.1(-) empty vector. In to tubes 2, 4, and 6, 2µg pCDNA3.1_miR-31 was added. Then I added 10µL Superfect reagent to each tube and the tubes were mixed. The tubes were then left at room temperature for 10 minutes. Meanwhile I prepared a 24 well plate of HeLa cells by removing the DMEM culture media and washing the wells with PBS.

After the 10-minute incubation of the tubes, I added 700µL DMEM culture media to each tube. Then I added 400µL from each tube to two wells to form duplicate samples, so that 12 wells were used (see Figure 8). I then incubated this plate for 3 hours at 37^{o}c. The supernatant was then removed, and I washed the cells with PBS. Then I added 500µL DMEM culture media to each well. The plate was then incubated overnight for 18 hours at 37^{o}c.

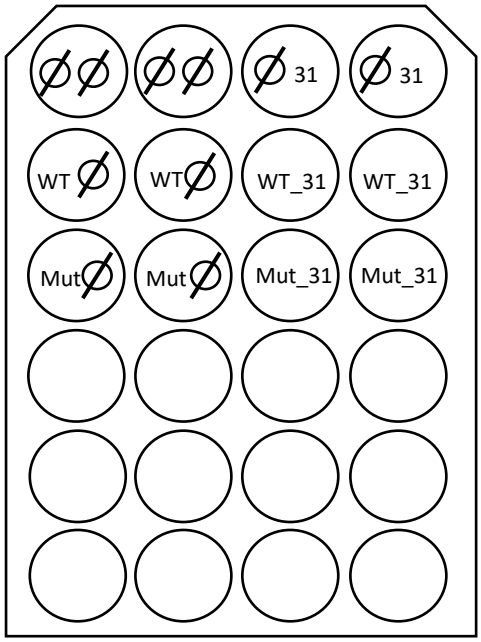


Figure 8. Plate layout for HeLa cell transfection

Following the overnight culture, I lysed the cells by removing the supernatant from the wells and washing the cells with PBS. Lysis buffer was then diluted, and I added 50µL of this to each well. The plate was then agitated for 20 minutes.

The supernatant from each well was removed and placed in to 1.5mL reaction tubes. I then centrifuged the samples at 13000g to pellet the debris. 20µL of each sample supernatant was added to 50µL aliquots of Luciferase reagent and I measured the samples on a Luminometer. Then I added 50µL of Stop and Glo and a further reading was taken on the Luminometer. I then created a ratio of the two readings to calculate the relative luminescence of each sample.

2.17 Statistics

I performed statistical analysis with GraphPad Prism for Windows version 5.0. Tables were generated using Microsoft Office Excel 2013 software.

Unpaired samples were assumed to have non-parametric distribution and therefore medians comparisons were analysed using the Mann-Witney U Test. Results were presented as distribution points, and a standard error from the mean.

Paired samples from the ex vivo model and TSLP ELISA were analysed using the two-tailed paired T-Test. The paired samples were represented by points and adjoining lines, or in the case of the TSLP ELISA as a box and whisker plot.

I analysed the data from the Tralokinumab study using the paired and unpaired T-Tests. When comparing the data in the groups of clinical responders to assess prediction to response the paired T-Test was applied. When comparing the data across the groups to assess baseline gene expression the unpaired T-Test was applied.

The luciferase experiment results are represented as a bar chart and statistical analysis applied using the two-tailed paired T-Test.

Regression analyses were used for comparing the relationships among variables such as microRNA expression and independent clinical indicators, and microRNA and target gene expression. The non-parametric Spearman correlation coefficient was used to calculate statistical dependence between the two variables, whether the variables were positively and negatively correlated. We were mindful to note that the correlation between variables did not prove causation, and in this circumstance further experiments would be carried out to investigate a causative relationship were performed.

Due to the small sample sizes in some of the experiments, interpretation of the readouts of the regression analysis and differences between the group demographics and clinical indicators was not different however with bigger sample sizes there could have been statical differences.

The clinical data has been represented in tables and the groups are statistically equal unless otherwise indicated.

A p value ≤ 0.05 is considered statistically significant.

3. Results

3.1 The effects of treatments used in inflammatory bowel disease in vivo on the expression of miR-31, miR-146a, and miR-155, and the expression of CCL18, SOCS1, and TNFα.

3.1.1 Introduction

Inflammatory bowel disease is a relatively new area of research with regard to the role microRNAs have to play in the pathophysiology. 4 microarray studies have shown 72 microRNAs are dysregulated in Ulcerative Colitis and Crohn's disease(*164, 169, 172, 206*). These studies show very few microRNAs with dysregulation across all the studies. One confounder for this data may be that the treatment which the patients were on was not taken into account. The purpose of this experiment is to stratify the patients in to groups of specific therapies, to see if the treatment itself could have an impact on the expression of specific microRNAs and cytokines. Another factor that may have influenced the data in these studies is the region of the colon that was biopsied. There is some debate as to whether microRNAs are differentially expressed at different sites around the colon and this factor was not taken in to account.

Patients were recruited into the study when they were having endoscopy as part of their routine clinical care, and all samples were from endoscopically affected sigmoid and terminal ileum mucosa, or in the case of the control group normal sigmoid and terminal ileum mucosa. For logistical reasons the histological assessment was not taken in to account. The patients were stratified into treatment groups on monotherapy with 5-Aminosalicylic Acid, thiopurine, Infliximab, and adalimumab, as well as active and quiescent untreated samples and normal healthy mucosa who were attending endoscopy for either polyp follow up, cancer follow up, or iron deficiency anaemia without a change in bowel habit.

At this time, we were unable to measure drug levels to assess drug efficacy. Patients on multiple or other immunotherapy, or steroids (whether that be for IBD or an alternative pathology) were excluded from the study. In the normal control group patients attending

with diarrhoeal symptoms or suspected IBD were also excluded. Azathioprine metabolite levels and anti-TNF drug levels can establish if adequate of effective drug therapy was being administered to these patients, although this is now evolving as part of routine clinical practice, we were unable to use these tests.

The patients were scored according to the mayo endoscopic score for ulcerative colitis, or the Crohn's disease activity index, by myself as an observer and the endoscopist performing the procedure. We were not blinded to each other's score so there is the problem of inter-observer variability which has not been overcome.

The microRNAs that were analysed had been shown to be dysregulated by microarray analyses previously performed by our group. A panel of three cytokines (CCL18, SOCS1, and TNFα) thought to be regulated by these microRNAs were also analysed. Our group has previously shown miR-155, miR-31, and miR-146a to be the microRNAs of interest in microarray analyses(*198, 199, 207*) and that by targeting genes with in the IL-13 pathway, there could be an association with the cytokine expression profiles, therefore these were chosen for more detailed analysis. Reverse Transcription PCR and RTqPCR was performed to analyse the expression of these genes and microRNAs.

Our hypothesis is that the different treatments used in IBD will have an effect on the expression profiles of these microRNAs and cytokines, and may lead to further insights into the mechanism by which they act.

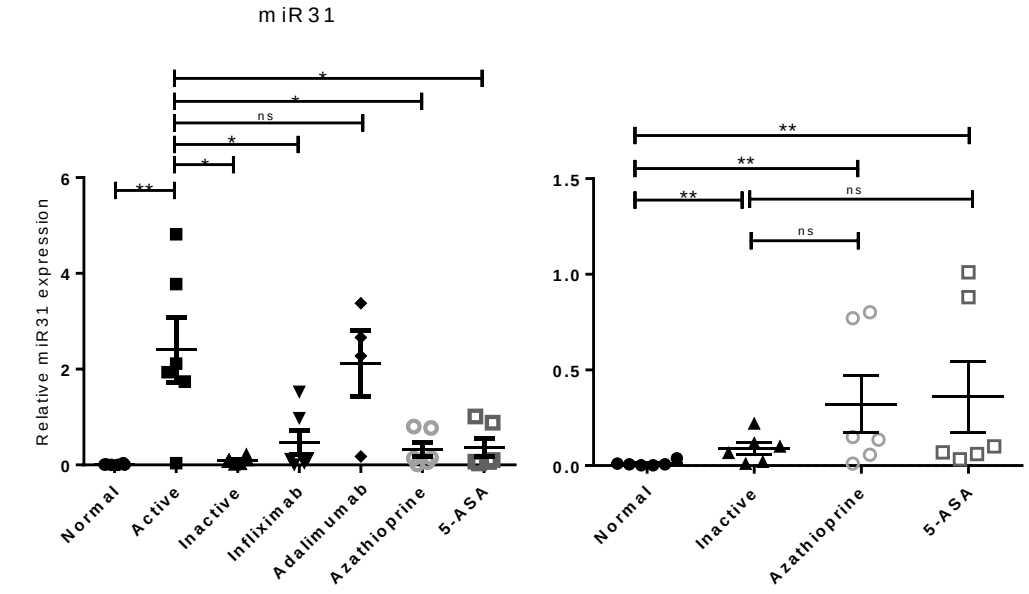
3.1.2 Ulcerative Colitis

3.1.2.1 Patient data.

	Normal n=6	Active UC n=6	Inactive UC n=6	Infliximab n=6	Adalimumab n=4	Azathioprine n=6	5-ASA n=6
Age	64 (49-86)	58 (32-77)	63 (44-81)	43 (28-58)	45 (28-60)	59 (32-79)	60 (31-82)
Sex M/F	2/4	4/2	4/2	3/3	1/3	4/2	3/3
Smoker	0	0	0	2	1	0	0
Disease duration (months)	0 (0-0)	22 (0-117)	222 (7-502)	28 (12-62)	17 (6-25)	245 (28-480)	155 (44-341)
Treatment time (months)	0 (0-0)	0 (0-0)	0 (0-0)	15 (6-24)	13 (6-24)	124 (24-240)	155 (44-341)
Mayo Score	0 (0-1)	11 (9-12)	1 (0-3)	1 (0-2)	1 (0-2)	0 (0-1)	1 (0-2)
Truelove and Witts	6 normal	5 severe 1 moderate	3 normal 3 mild	4 normal 2 mild	2 normal 2 mild	6 normal	5 normal 1 mild
Haemoglobin	136 (111-152)	98 (77-126)	116 (86-140)	130 (117-150)	130 (111-145)	120 (84-137)	134 (122-147)
CRP	6 (0-12)	64 (22-146)	6 (0-22)	12 (2-32)	6 (0-14)	8 (0-24)	12 (0-46)
Albumin	40 (36-45)	31 (20-41)	35 (17-50)	39 (35-43)	40 (36-44)	38 (36-40)	37 (28-40)

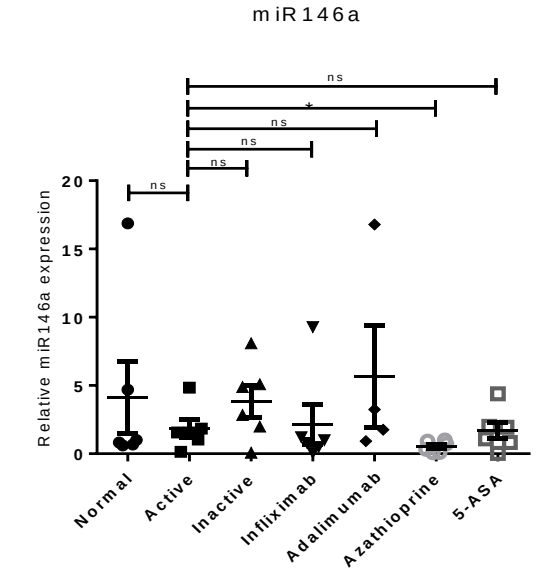
Table 22. Patient demographics, clinical scores and clinical indices. Active UC was defined as a Mayo score of 6 or greater. Inactive disease was defined as Mayo score of 0-3. The drug treatments represent patients with a clinical response, and a Mayo score of 0-3

3.1.2.2 microRNA expression profiles are altered by different treatment in ulcerative colitis



Mann-Whitney U test
* - p<0.05
** - p<0.01

Figure 9. The effect of treatment on miR-31 expression in UC. n=6 in each treatment group. The second graph represents the original graph without the biological therapies. Only Azathioprine and 5-ASA reduce miR-31 expression



Mann-Whitney U test
* - p<0.05
** - p<0.01

Figure 10. The effect of treatment on miR-146a expression in UC. n=6 in each treatment group. None of the treatments significantly alter miR-146a expression

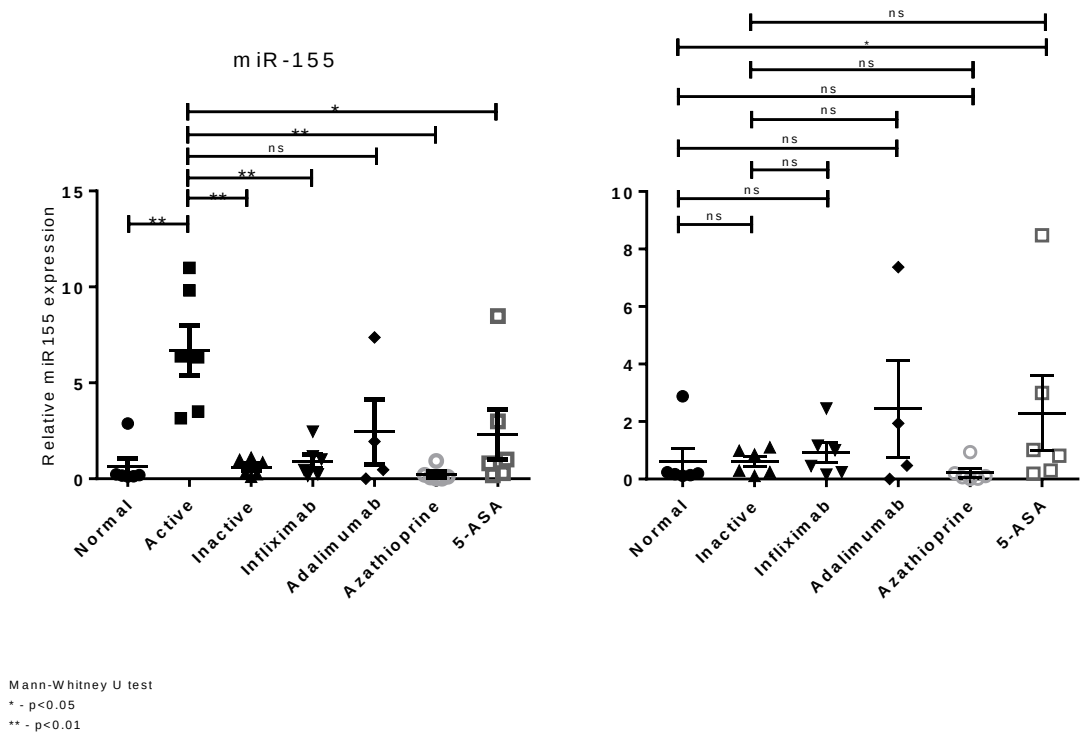


Figure 11. The effect of treatment on miR-155 expression in UC. n=6 in each treatment group. Adalimumab is the only treatment that does not significantly reduce miR-155 expression compared to actively diseased mucosa

I have confirmed that miR-31 expression is significantly raised in patients with untreated active and inactive UC when compared to normal mucosa. Furthermore, patients with untreated active UC have significantly raised miR-31 expression when compared to the patients with untreated inactive disease. When patients are treated with azathioprine and 5-ASA and achieved good mucosal healing endoscopically using the mayo score, the miR-31 reduced down to that of quiescent disease. However, the anti-TNF therapies did not reduce down to the level of untreated inactive disease, in fact the Adalimumab group did not have a significantly reduced miR-31 level when compared to the active group despite the endoscopic response seen.

This data shows miR-146a expression is not altered in Ulcerative colitis. There is no significant difference in the expression of miR-146a when comparing the sigmoid mucosa of untreated active and inactive UC with that of normal mucosa. The treatment groups again show no statistical difference in the expression of miR-146a except for Azathioprine in which there is a significant decrease in miR-146a expression, although the clinical relevance of this is debatable given the expression seen in normal healthy mucosa.

I confirm that miR-155 expression is significantly increased in the sigmoid mucosa of untreated active UC when compared to that of untreated inactive UC and normal healthy mucosa. There is no significant difference between inactive UC and normal healthy mucosa, although the trend is for inactive disease to have increased expression. All the treatment groups here reduce the expression of miR-155, except Adalimumab, although there is no statistical difference between this group and the inactive and normal groups. The group treated with 5-ASA still have significantly increased miR-155 expression when compared to healthy mucosa, however the other treatments reduce the expression down to levels seen in normal healthy mucosa.

3.1.2.3 Cytokine expression profiles are altered by different treatment in ulcerative colitis

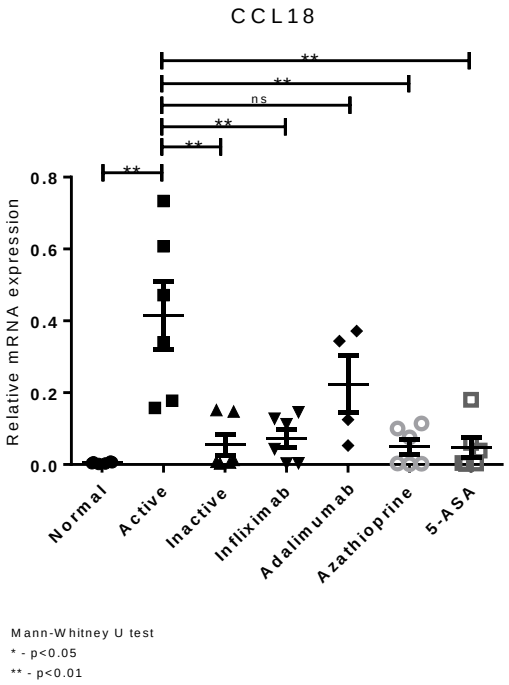


Figure 12. The effect of treatment on CCL18 expression in UC. n=6 in each treatment group. CCL18 expression is not significantly reduced by Adalimumab

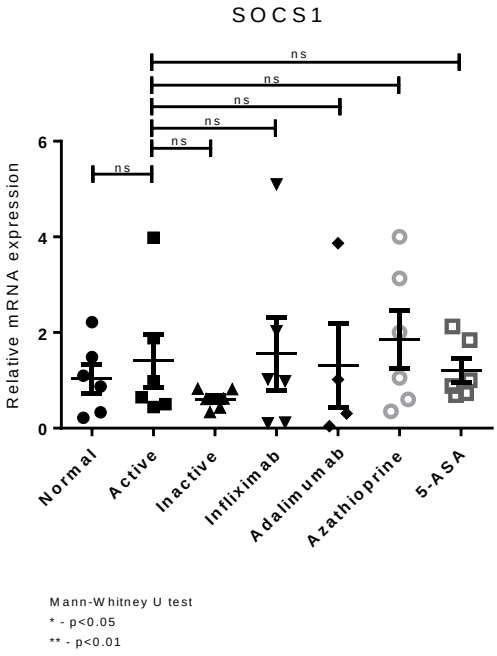


Figure 13. The effect of treatment on SOCS1 expression in UC. n=6 in each treatment group. SOCS1 expression is not altered by IBD treatments

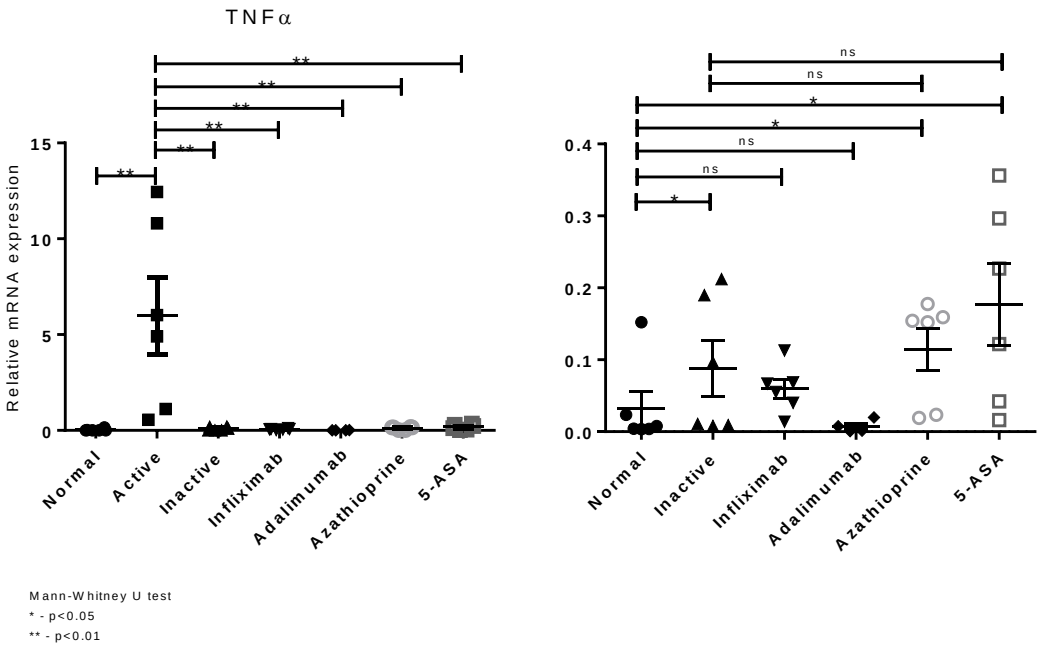


Figure 14. The effect of treatment on TNFα expression in UC. n=6 in each treatment group. All treatments reduce TNFα expression from actively diseased mucosa. The second graph is the same data with the active group removed to allow for easier illustration of results and analysis.

The expression of CCL18 is significantly increased in the mucosal biopsies of untreated active UC when compared to untreated inactive UC and normal healthy sigmoid mucosal biopsies. The expression of CCL18 is not significantly increased in untreated inactive disease when compared to normal mucosa. All the therapies reduce the CCL18 expression down in equal measure to that of untreated inactive disease except the anti-TNF therapy Adalimumab, although Infliximab does achieve down regulation of CCL18 to inactive disease levels. There is no statistical difference of CCL18 expression between normal healthy mucosa and untreated inactive UC, however the trend suggests the expression of CCL18 is lower in normal mucosal biopsies.

There is no difference in the expression of SOCS1 in UC, whether untreated active or inactive and regardless of the treatment regimen. Furthermore, SOCS1 expression in sigmoid mucosa is no different when comparing untreated active and inactive UC to normal healthy mucosal biopsies.

As you can see in the Figure untreated active UC as you might expect has significantly increased expression of TNFα when compared to untreated inactive disease, normal healthy

mucosa, and all the treatment responder groups. inactive UC has upregulated TNFα expression when compared to normal mucosa. Anti-TNF therapies down regulate the expression of TNFα to that of normal healthy mucosa, whereas 5-ASA and Azathioprine therapy reduce the expression to that of an untreated inactive disease state.

3.1.3 Colonic Crohn's Disease

3.1.3.1 Patient Data

	Normal n=6	Active Crohn's n=7	Inactive Crohn's n=6	Infliximab n=7	Adalimumab n=6	Azathioprine n=6	5-ASA n=6
Age	64 (49-86)	31 (17-56)	41 (23-56)	28 (22-45)	36 (17-49)	40 (23-51)	43 (24-61)
Sex M/F	2/4	5/2	4/2	5/2	3/3	1/5	3/3
Smoker	0	1	2	0	1	2	1
Disease length (months)	0 (0-0)	105 (0-537)	196 (33-525)	80 (3-241)	137 (40-320)	169 (18-520)	174 (1-360)
Treatment time (months)	0 (0-0)	0 (0-0)	0 (0-0)	18 (3-36)	20 (12-36)	66 (12-120)	112 (1-220)
CDAI	66 (0-134)	337 (221-432)	94 (38-209)	85 (12-149)	91 (20-142)	118 (90-142)	66 (0-121)
Harvey Bradshaw Index	3 (0-5)	13 (8-18)	2 (0-6)	2 (0-5)	3 (1-5)	4 (2-5)	1 (0-4)
Haemoglobin	136 (111-152)	101 (81-115)	139 (128-152)	139 (125-158)	139 (126-160)	138 (122-152)	128 (116-146)
CRP	6 (0-12)	98 (44-224)	14 (0-66)	5 (0-24)	4 (0-12)	4 (0-13)	8 (0-32)
Albumin	40 (36-45)	28 (17-40)	41 (38-44)	41 (33-49)	37 (22-42)	37 (28-41)	40 (38-40)

Table 23. Patient demographics, clinical scores and clinical indices. Active CD was defined as a CDAI score of 150 or greater, or HBI greater than 5. Inactive disease was defined as CDAI of 0-150, or an HBI of 0-5. The drug treatments represent patients with a clinical response with a CDAI of less than 150, CDAI fall of over 70, or an HBI of 0-5

3.1.3.2 microRNA expression profiles are altered by different treatment in Crohn's colitis

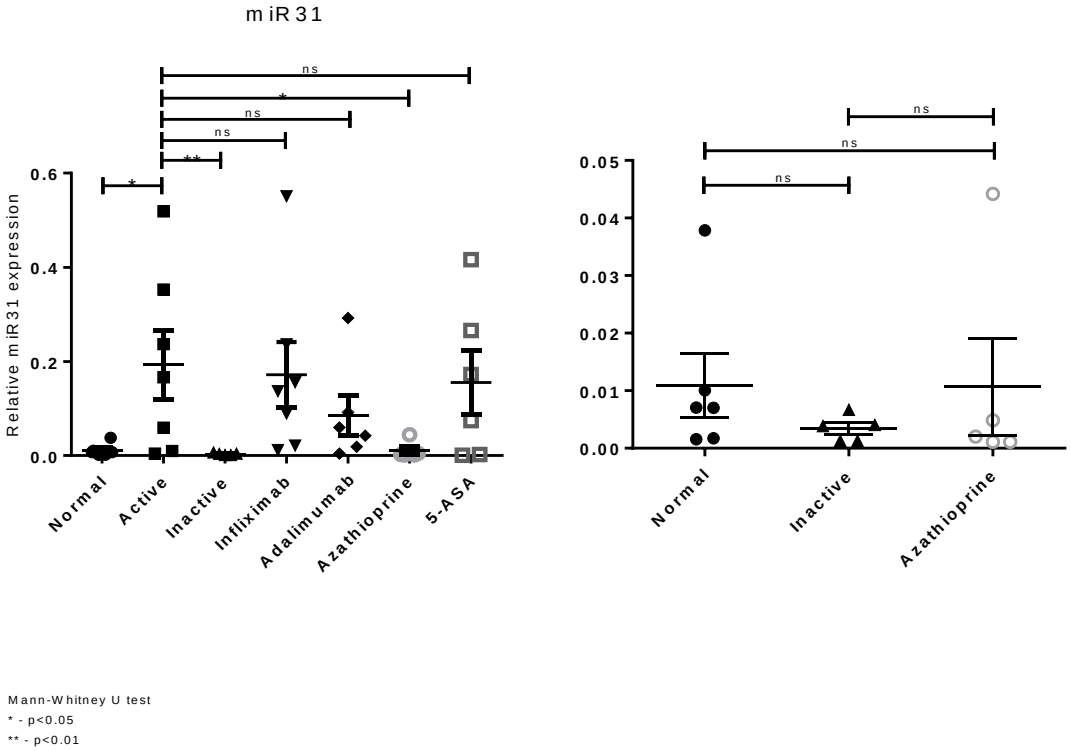


Figure 15. The effect of treatment on miR-31 expression in Colonic Crohn's Disease. n=6 in each treatment group. Only Azathioprine reduces miR-31 expression significantly in CD

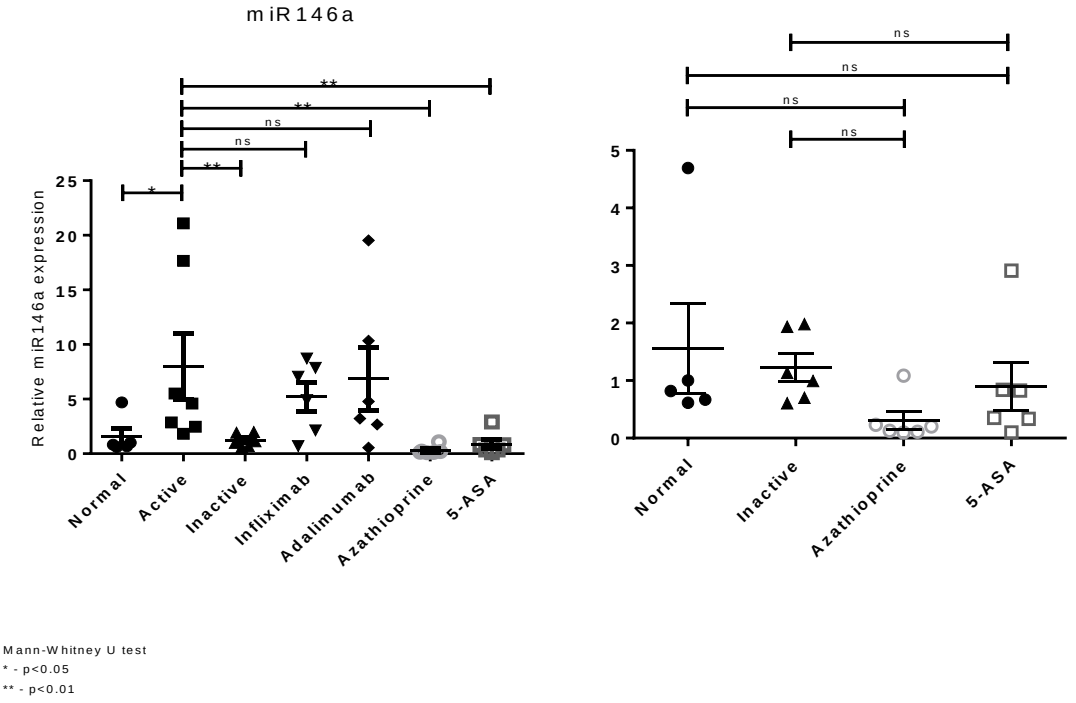


Figure 16. The effect of treatment on miR-146a expression in Colonic Crohn's Disease. n=6 in each treatment group. Only Azathioprine and 5-ASA reduce miR-146a expression significantly in CD

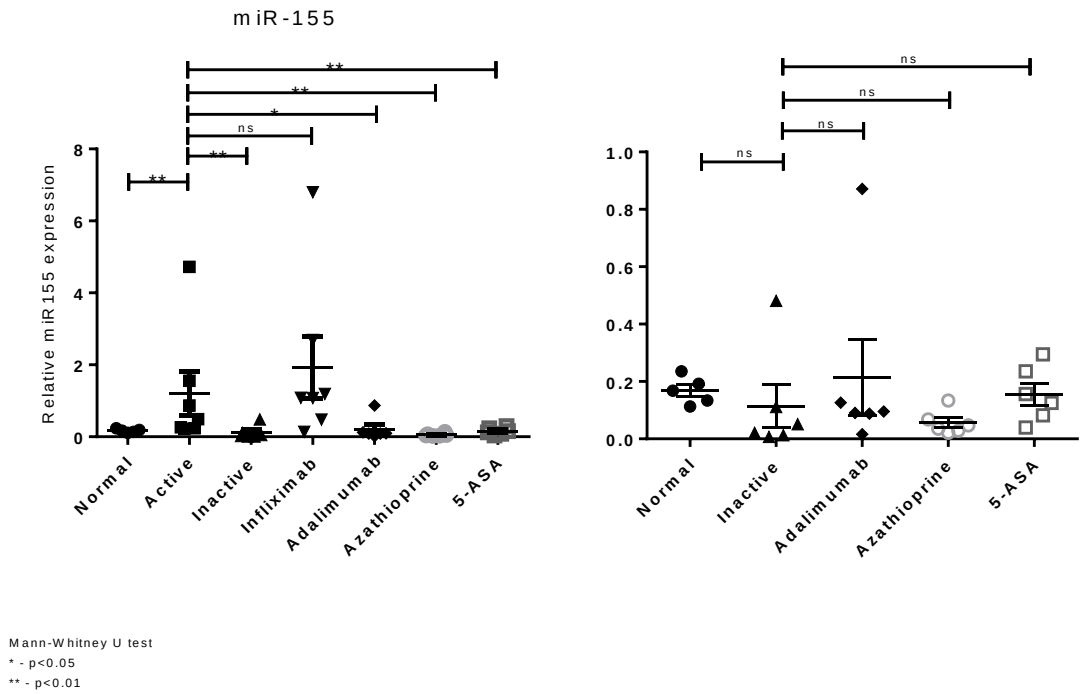


Figure 17. The effect of treatment on miR-155 expression in Colonic Crohn's Disease. n=6 in each treatment group. Only Infliximab does not reduce miR-155 expression significantly in CD

As seen in UC the expression of miR-31 is significantly higher in untreated active sigmoid Crohn's disease when compared to inactive sigmoid mucosal biopsies, and normal healthy sigmoid mucosa. In untreated inactive Crohn's colitis miR-31 is not expressed more when compared to normal sigmoid mucosa. Despite being endoscopically treated, the only treatment that significantly lowers the miR-31 expression was Azathioprine, in fact in the Azathioprine treated mucosal biopsies miR-31 expression was lowered to that of normal healthy sigmoid mucosa and untreated inactive colonic Crohn's disease. At this stage it cannot be determined whether this is due to a mechanism of action of Azathioprine, or whether it was due to factors within the patient selection such as disease severity with higher starting miR-31 expression, or time since treatment was initiated.

Unlike UC, miR-146a expression is significantly higher in the sigmoid mucosa of untreated active Crohn's colitis when compared to untreated inactive sigmoid Crohn's colitis and normal healthy sigmoid biopsies. Untreated inactive sigmoid Crohn's colitis does not have increased miR-146a expression when compared to normal healthy mucosal biopsies. In the endoscopic remission groups, the two anti-TNF therapies did not significantly lower the expression of miR-146a. However, as seen with miR-31, azathioprine does lower the expression of miR-146a to that of levels seen in both inactive sigmoid Crohn's disease, and

normal mucosal biopsies. Unlike what was seen with miR-31, 5-ASA therapy also appears to lower the expression of miR-146a in the mucosa. Again, this could be because of a mechanism by which these drugs act or because of the initial severity of the disease state in the anti-TNF treated population or time of drug exposure.

miR-155 expression is significantly increased in the mucosa of untreated active Crohn's colitis but not in the mucosa of untreated inactive Crohn's colitis when compared to miR-155 expression in normal mucosal biopsies. All the treatment groups with endoscopic remission have down regulated miR-155 expression except for Infliximab. The level of miR-155 expression in the treatment groups is statistically similar to the mucosal miR-155 expression seen in both normal mucosa in untreated inactive Crohn's colitis.

3.1.3.3 Cytokine expression profiles are altered by the treatments in Crohn's colitis

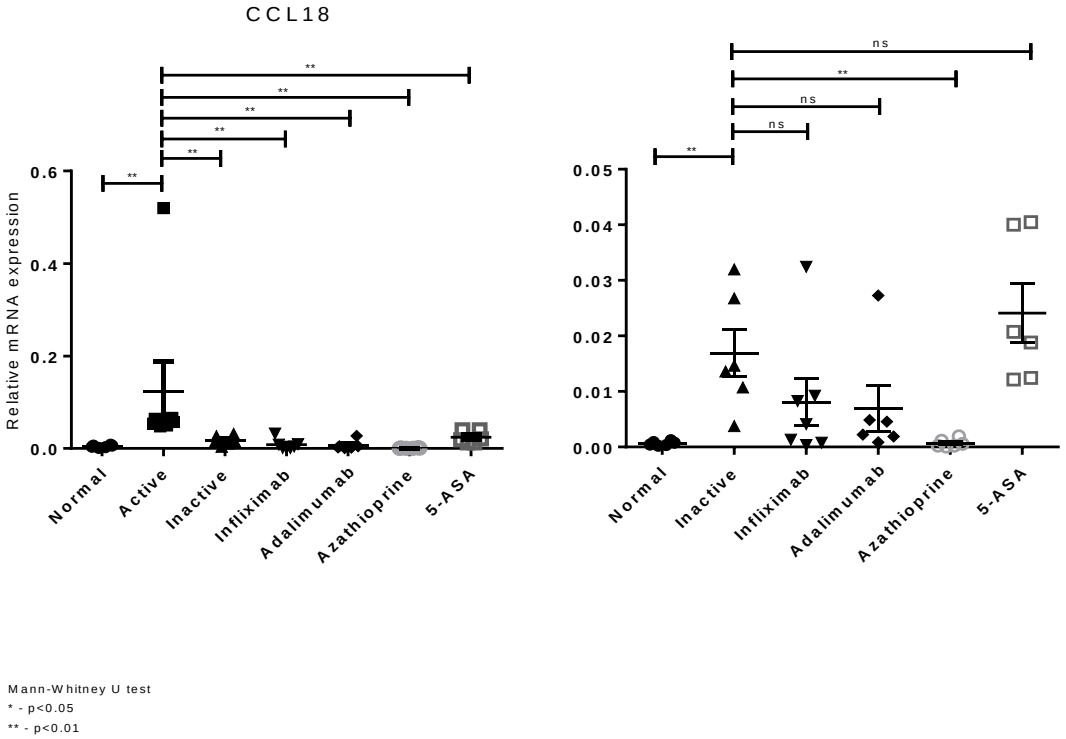


Figure 18. The effect of treatment on CCL18 expression in Colonic Crohn's Disease. n=6 in each treatment group. All treatments reduce CCL18 expression significantly in CD. The second graph has the active group removed to allow for easier illustration of results and analysis.

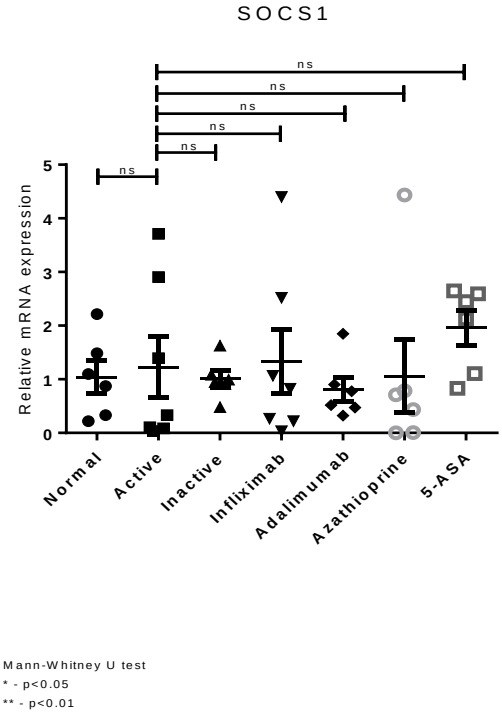


Figure 19. The effect of treatment on SOCS1 expression in Colonic Crohn's Disease. n=6 in each treatment group. No treatments reduce SOCS1 expression significantly in CD

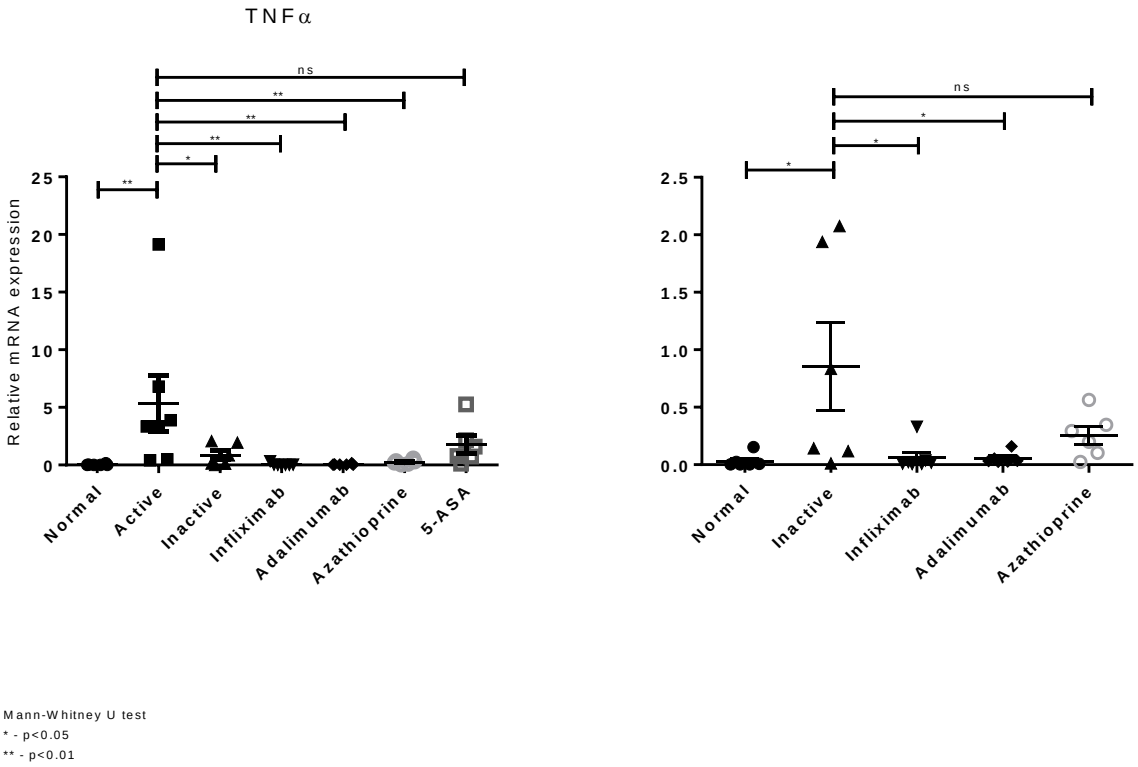


Figure 20. The effect of treatment on TNFα expression in Colonic Crohn's Disease. n=6 in each treatment group. All treatments except 5-ASA reduce TNFα expression significantly in CD

The expression of CCL18 in the mucosa of untreated active sigmoid Crohn's colitis is significantly increased when compared to untreated inactive sigmoid Crohn's colitis and normal healthy sigmoid mucosa. Inactive sigmoid mucosa has also increased CCL18 expression when compared to normal mucosa but to a lesser extent than that of untreated active Crohn's colitis. All the treatments significantly reduce the expression of CCL18 in the mucosa, however azathioprine is the only therapy that reduces the CCL18 expression down to similar levels in normal healthy mucosal biopsies.

As we saw in UC, there is no difference in the expression of SOCS1 in Crohn's Colitis, whether untreated active or inactive and regardless of the treatment regimen. Furthermore, SOCS1 expression in sigmoid mucosa is no different when comparing untreated active and inactive Crohn's Colitis to normal healthy mucosal biopsies.

As you might expect, the expression of TNFα in significantly raised in untreated active sigmoid Crohn's colitis when compared to untreated inactive Crohn's colitis and normal healthy sigmoid mucosa. Inactive Crohn's colitis also has a significantly increased expression of TNFα when compared to normal healthy mucosa. Patients taking 5-ASA therapy do not

have significantly lower TNFα when compared to active disease, but the anti-TNF therapies, Infliximab and Adalimumab, and Azathioprine do lower the TNFα expression. Infliximab and Adalimumab lower the TNFα expression significantly more than untreated inactive disease, to levels seen in normal healthy mucosa.

3.1.4 Ileal Crohn's Disease

3.1.4.1 Patient Data

	Normal n=6	Active Ileitis n=7	Inactive Ileitis n=6	Infliximab n=6	Adalimumab n=6	Azathioprine n=6
Age	62 (45-81)	29 (17-48)	43 (23-75)	28 (20-47)	32 (20-44)	29 (18-45)
Sex M/F	3/3	4/3	2/4	2/4	4/2	3/3
Smoker	0	1	2	0	1	0
Disease duration (months)	0 (0-0)	38 (0-248)	59 (0-141)	62 (5-107)	121 (15-190)	50 (13-102)
treatment time (months)	0 (0-0)	0 (0-0)	0 (0-0)	23 (5-60)	27 (12-60)	45 (12-100)
CDAI	41 (0-86)	409 (288-565)	41 (0-89)	96 (40-145)	76 (0-140)	71 (0-142)
Harvey Bradshaw Index	1 (0-4)	14 (8-20)	1 (0-2)	4 (2-5)	2 (0-5)	2 (0-5)
Haemoglobin	134 (108-147)	107 (86-128)	136 (119-155)	129 (118-152)	139 (122-160)	137 (118-156)
CRP	8 (0-36)	70 (0-143)	18 (0-79)	15 (0-38)	3 (0-9)	5 (0-12)
Albumin	37 (30-41)	30 (26-38)	40 (33-44)	41 (33-49)	36 (22-44)	38 (30-42)

Table 24. Patient demographics, clinical scores and clinical indices. Active CD was defined as a CDAI score of 150 or greater, or HBI greater than 5. Inactive disease was defined as Mayo score of 0-150, or an HBI of 0-5. The drug treatments represent patients with a clinical response with a CDAI of less than 150, CDAI fall of over 70, or an HBI of 0-5

3.1.4.2 microRNA expression profiles are altered by different treatment in Crohn's ileitis

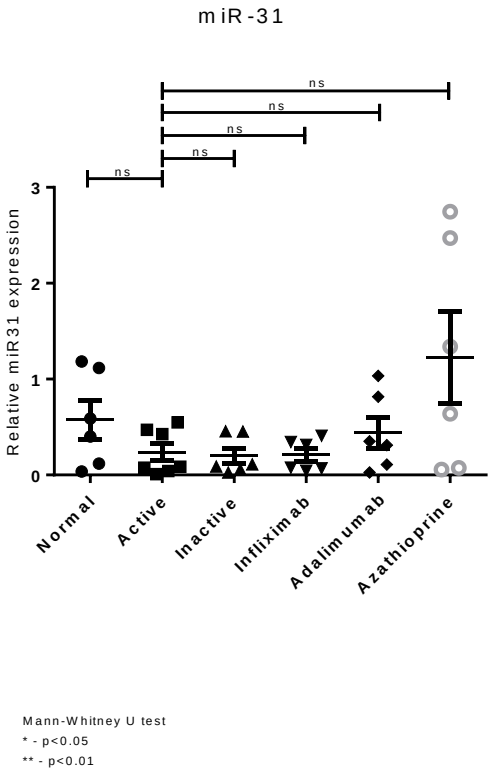


Figure 21. The effect of treatment on miR-31 expression in Ileal Crohn's Disease. n=6 in each treatment group. miR-31 expression is not altered in TI CD

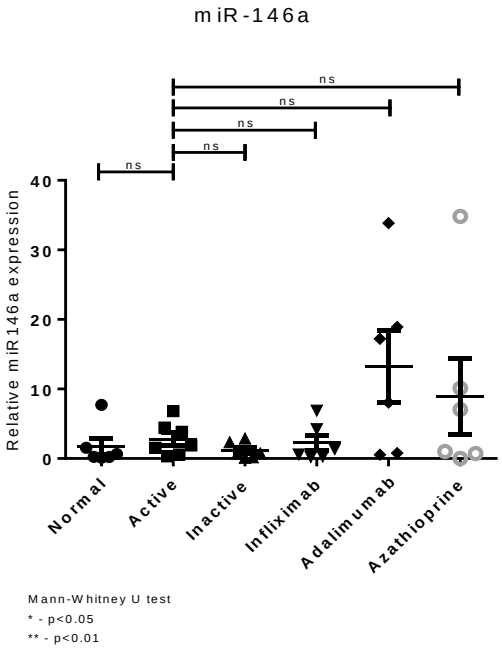


Figure 22. The effect of treatment on miR-146a expression in Crohn's ileitis. n=6 in each treatment group. miR-146a expression is not altered in TI CD

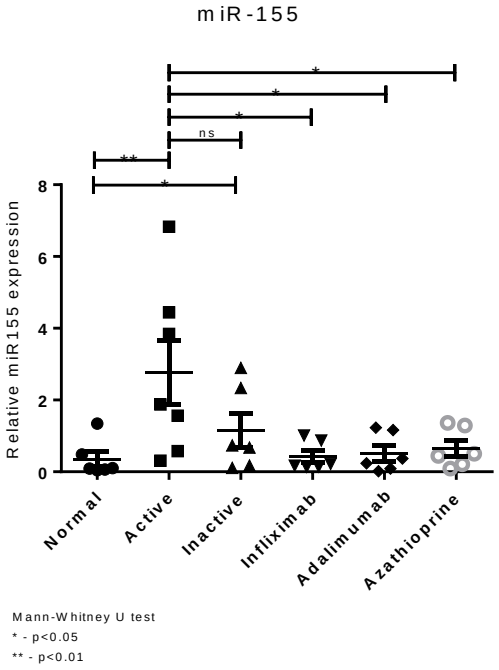


Figure 23. The effect of treatment on miR-155 expression in Crohn's ileitis. n=6 in each treatment group. miR-155 expression is reduced by all treatments in TI CD

Unlike what we have observed in colonic Crohn's disease, and UC, miR-31 expression is not upregulated in untreated active ileitis when compared to untreated inactive ileal disease or normal ileal mucosa. In fact, there is a trend for the opposite, with decreased miR-31 expression in untreated active and inactive Crohn's ileitis when compared to normal ileum. None of the therapies alter the miR-31 expression significantly within the ileal mucosa, however, patients taking Azathioprine do have increased mir-31 expression in the ileal mucosa compared to the other therapies.

Unlike colonic Crohn's disease, miR-146a expression is not upregulated in untreated active Crohn's ileitis when compared to untreated inactive ileal disease and normal ileal mucosa. None of the therapies alter the miR-146a expression significantly within the ileal mucosa, or though there is a trend seen in the Adalimumab and Azathioprine treated groups, for upregulated miR-146a expression.

miR-155 expression is increased in the ileum of both untreated active and inactive terminal ileal Crohn's disease when compared to normal ileal mucosa. Inactive Crohn's ileitis has less of an up regulatory response to miR-155 expression than active Crohn's ileitis. Both the anti-TNF therapies and Azathioprine reduce the miR-155 expression down to the level observed in normal ileal mucosa. This could suggest that inactive disease should still be treated as

there may be sub endoscopic inflammation occurring. This could have implications for the use of maintenance therapy in CD given that treatment decisions are often heavily influenced by the macroscopic appearances at endoscopy.

3.1.4.3 Cytokine expression profiles are altered by different treatment in Crohn's ileitis

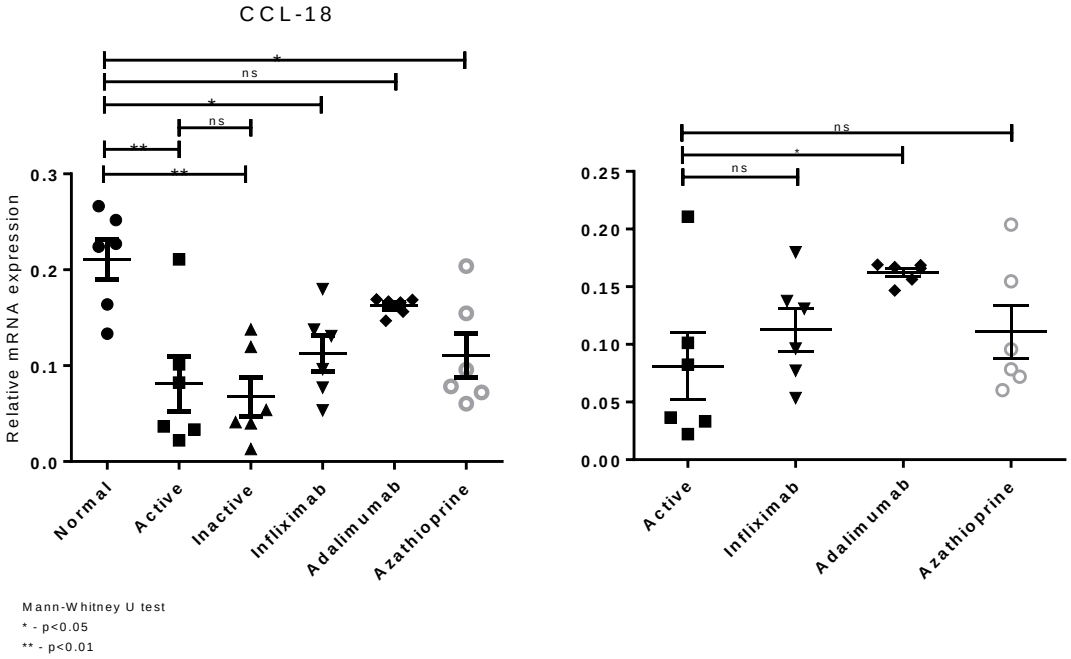


Figure 24. The effect of treatment on CCL18 expression in Crohn's ileitis. n=6 in each treatment group. CCL18 expression is reduced in TICD, and only Azathioprine up-regulates CCL18 expression

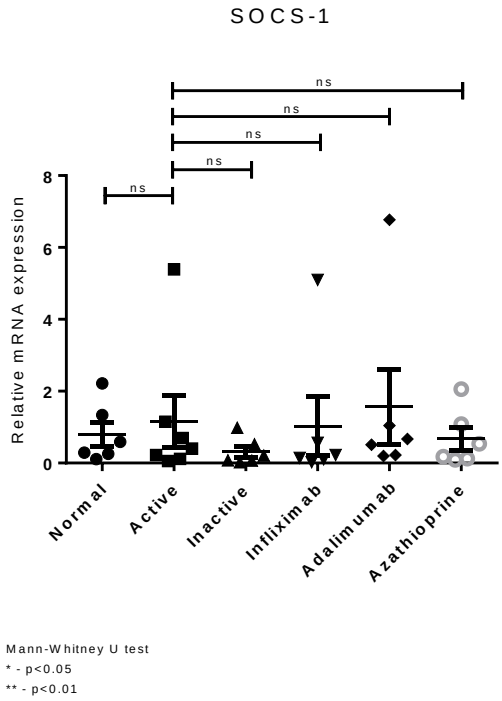


Figure 25. The effect of treatment on SOCS1 expression in Crohn's ileitis. n=6 in each treatment group. SOCS1 expression is not altered in TICD

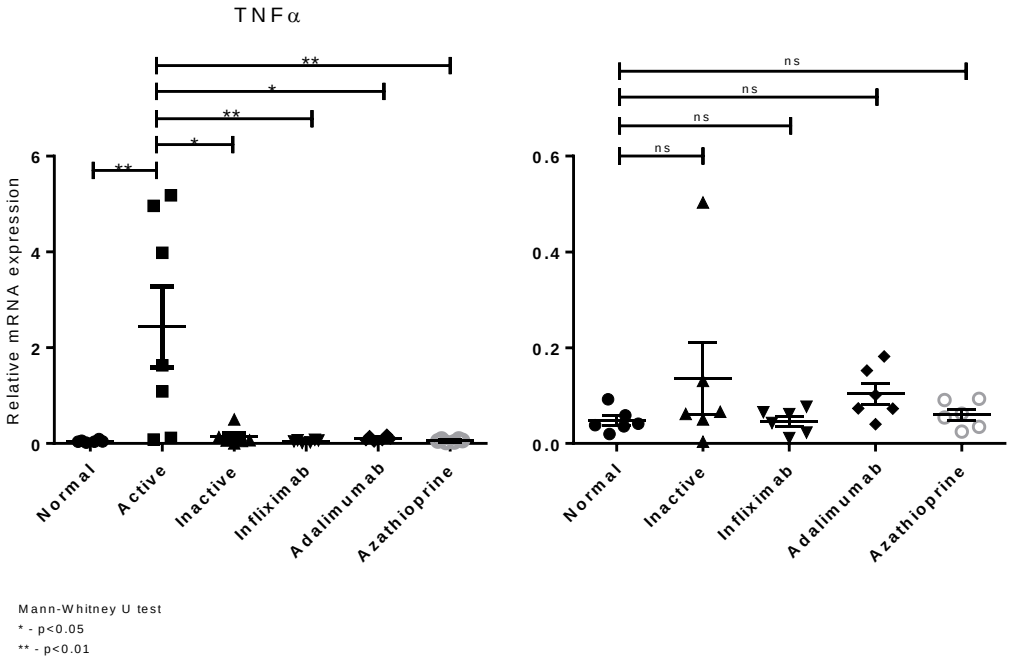


Figure 26. The effect of treatment on TNFα expression in Crohn's ileitis. n=6 in each treatment group. TNFα expression is reduced by all treatments in TICD

Interestingly the CCL18 expression seen in mucosal biopsies of untreated active and inactive ileal Crohn disease is reduced when compared to normal healthy ileal biopsies. This is in contrast to increased expression seen in untreated colonic Crohn's disease and Ulcerative colitis. The patients on treatment for the disease do have increased CCL18 expression but only Adalimumab is the expression significantly increased, and statistically similar to the levels seen in normal ileal mucosa.

There is no difference in the expression of SOCS1 in Crohn's ileitis, whether untreated active or inactive and regardless of the treatment regimen. Furthermore, SOCS1 expression in the ileum is no different when comparing untreated active and inactive Crohn's ileitis to normal healthy mucosal biopsies.

Active untreated Crohn's disease of the ileum has significantly increased TNFα expression when compared to untreated inactive disease and normal ileal mucosa. Inactive Crohn's ileitis does not have increased expression when compared to normal mucosal biopsies. The anti-TNF therapies and Azathioprine all equally reduced the expression of TNFα down to the levels seen in inactive disease.

3.1.5 Discussion

I have shown here there is a differential expression of specific microRNAs and cytokines in active IBD, and that the treatments used alter this expression profile in different ways. I have also shown that the expression profiles vary between UC and Crohn's colitis, and more strikingly, between Crohn's disease of the ileum and colon.

The expression of miR-31 is significantly lower in the sigmoid mucosa of normal colon and inactive disease when compared to active disease in both UC and Crohn's colitis. There is also increased expression in the inactive sigmoid mucosa of UC patients when compared to normal mucosa, however this effect is not reciprocated in colonic Crohn's disease. In UC treatment with both 5-ASA and Azathioprine reduces the miR-31 expression compared to active disease, down to a level statistically insignificant from untreated inactive disease, yet still increased when compared to normal healthy mucosa. The anti-TNF treatments do not reduce miR-31 expression down as effectively despite the endoscopic and clinical scores indicating remission. It is possible that the initial disease severity was higher in these patients, hence the use of anti-TNF therapy, so the miR-31 expression was higher in these patients before treatment was commenced, however this cannot be confirmed. The patient data shows that the treatment course is shorter and therefore the reduced miR-31 expression in the long-term may reduce, but this effect is not seen in the shorter time span of the anti-TNF group. Alternatively, the miR-31 expression may be reduced by Azathioprine and 5-ASA as part of their mechanism of action, and anti-TNF therapy is not treating the disease using this pathway. A similar pattern is seen in Crohn's colitis; however, 5-ASA does not reduce the expression of miR-31 as it does in UC; the role of 5-ASAs is questionable in the treatment of Crohn's disease. Again, Azathioprine reduces the expression of miR-31, in fact the expression is reduced to a level statistically insignificant from normal colonic mucosal miR-31 expression. The anti-TNF therapies do not significantly reduce miR-31 expression, but again the treatment course in this group is shorter compared to immunomodulatory therapy.

In Crohn's ileitis, miR-31 expression is not increased when compared to normal Ileal mucosa, if anything the opposite is true. The expression of miR-31 is increased in normal ileum, when compared to normal colon. This effect is not seen when the tissues are actively

inflamed with Crohn's disease. Therefore miR-31 may not be playing as an important role in small bowel Crohn's disease. (Figure 21)

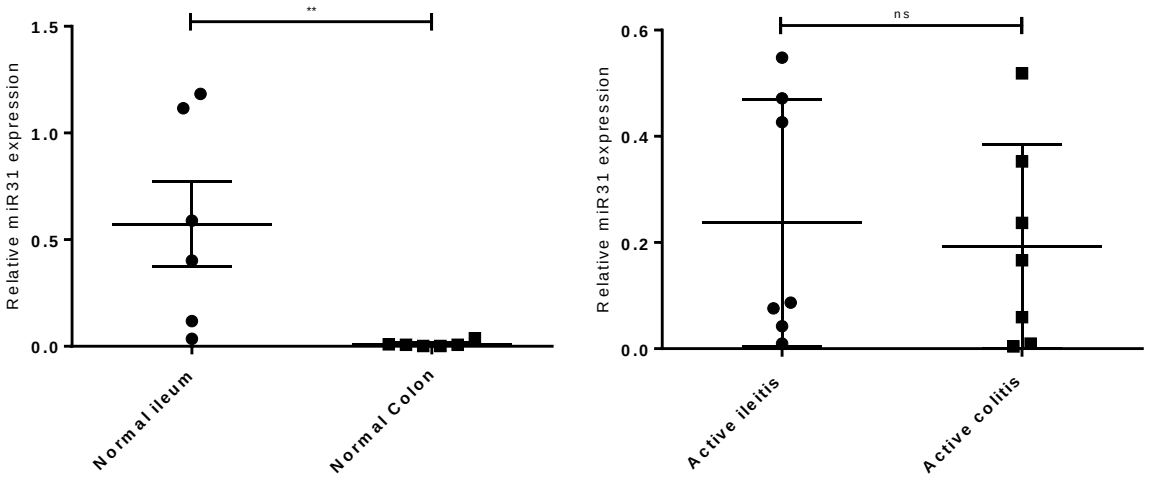


Figure 27. Comparing miR-31 expression in ileum and colon. Normal colonic miR-31 expression is reduced compared to normal ileum. But this effect is not seen when the tissues are actively inflamed

The treatment of Crohn's ileitis with anti-TNF therapy does not significantly alter the miR-31 expression in the ileum, when compared to active treatment naïve disease, however treatment with Azathioprine does seem to increase the miR-31 expression in the diseased ileum. This suggests differences in the miRNA influence on the mechanism of inflammation between colonic and ileal Crohn's disease, but also the way the drugs work to treat the disease may vary between drugs depending on the site of disease.

miR-31 indirectly controls the expression of CCL18, a marker of Th2 type inflammation(*208*). The pattern of CCL18 expression in colonic and ileal CD, and UC, and across the treatment groups, follows a similar pattern to miR-31 expression. This gives weight to the idea of miR-31 influencing this pathway. It also appears that this pathway is one of the mechanisms by which Azathioprine acts to treat IBD.

miR-146a expression does not change significantly in either colon or ileum whether it be diseased with Crohn's or UC, or normal healthy mucosa. It is noted that Azathioprine does reduce miR-146a expression significantly in the colon. This effect is not seen in the ileum though miR-146a expression is increased. The effect Azathioprine is having on miR-146a expression, by seemingly reducing miR-146a expression, may not be therapeutic. The expression in normal mucosa is not significantly different to expression in active disease, regardless of the tissue analysed.

miR-155 is known to target genes involved in TNFα expression. It is thought that increased miR-155 will increase the expression of TNFα by targeting TNFα suppressor genes. As you can see TNFα expression is increased in active treatment naïve IBD irrespective of classification or site. Treating IBD reduces this expression in line with clinical response as assessed by clinical and endoscopic criteria. As you might expect the anti-TNFα biological therapies are most effective at doing this. The effect this has on miR-155 expression is generally to also decrease its expression. However, this effect is less marked in the anti-TNF therapies. This could be that these therapies are targeting TNFα directly, thus causing initial miR-155 increased expression as a result, before the reduced inflammatory response can cause a decreased miR-155 expression as part of a feedback loop. Thiopurines and 5-ASA drugs may reduce inflammation by other pathways and thus miR-155 expression is reduced as inflammation reduces. As a result, TNFα expression decreases too. It is interesting to note that 5-ASA therapy is less effective at reducing miR-155 and TNFα expression in Crohn's colitis when compared to UC which is consistent with the clinical the clinical response of 5-ASA being less effective in Crohn's disease.

In general Crohn's colitis has a similar profile in it microRNA and cytokine expression to UC rather than Crohn's ileitis. This could be due to the underlying tissue that is diseased, but also that colonic and ileal disease have different immunological processes and are in fact different diseases. Clinically this would fit as the colonic and small bowel phenotypes are very different.

One weakness is that the patients were scored endoscopically by myself as an observer and the endoscopist performing the procedure. We were not blinded to each other's score so there is the problem of inter-observer variability which has not been overcome. The scoring systems of the mayo endoscopic score for ulcerative colitis, or the Crohn's disease activity index, also have their own limitations but are the most appropriate in the use for this type of research.

Regression analysis comparing cytokine and microRNA reading with patient factors such as age, smoking or endoscopic scores and biochemical reading did not show any statistical relationships. Examples of this have been shown in appendix 3.

3.2 An Ex Vivo model to show the effect of treatments in inflammatory bowel disease on the expression of miR-31, miR-146a, and miR-155, and the expression of CCL18, SOCS1, and TNFα.

3.2.1 Introduction

In the previous section, I showed that the different treatments can affect the expression of cytokines and microRNAs in the different forms of IBD to varying extents. However, these samples were unpaired so direct comparison between expression profiles before and after treatment are difficult to make. This is especially true when comparing the treatment groups to each other as the patients having anti-TNFα therapy are like to have more severely active inflammation before treatment was started than patients treated with 5-ASA. In this study I used treatment naïve tissue from patients with actively inflamed ulcerative colitis, Crohn's colitis, and Crohn's Ileitis as well as normal colonic mucosa, and treat the biopsies with all the therapies separately. This will provide evidence that the change in microRNA and cytokine expression is a result of the specific treatments and not due to patient stratification differences between the treatment groups.

Again, the patients were scored according to the mayo endoscopic score for ulcerative colitis, or the Crohn's disease activity index, by myself as an observer and the endoscopist performing the procedure. We were not blinded to each other's score so there is the problem of inter-observer variability which has not been overcome.

Six biopsies were collected in Aqix© cell culture medium from each patient. The viable biopsies were then incubated with either Aqix© alone or with Aqix© and a treatment as discussed in the methods (chapter 2.11). 5-ASA was prepared in DMSO, so a DMSO control group was also incubated and analysed. For the purposes of the experiment 6-Thioguanine was used as a Thiopurine analogue. This is the active metabolite of Azathioprine and 6-Mercatopurine, the drugs used in patient treatment. The tissue is viable for this incubation period and therefore ex-vivo treatment of these biopsies could be achieved by adding the drugs to the culture media. After the incubation period the biopsies were homogenised in

Trizol© and the RNA was extracted using Direct-zol™ RNA miniprep columns and expression analysed using RTqPCR.

Human ex-vivo models have already been successful in lung tissue when looking at response to viral or cytokine exposure in asthma and chronic obstructive pulmonary disease(*209, 210*). Using these models as examples I have transferred this into a model for IBD.

Here we show how treatments affect the cytokine and microRNA expression in active inflammatory bowel disease in paired samples. This eliminates the variation in treatment duration and initial disease severity between the pre and post treatment samples. The control samples in each group are biopsies taken at the same time but incubated without any drug. Significance here is measured using the paired T-Test ($p<0.05$).

This data could then be used to show that the microRNA profiles prior to treatment may not only aid the diagnosis between UC and Crohn's colitis, but also give us predictive biomarkers, predicting which drugs may or may not be of benefit in specific patients. This could stop unnecessary use of drugs that are unlikely to benefit certain patients, reducing exposure to unnecessary side effects and reducing healthcare costs, but also introduce personalised medicine and improve patient outcomes.

Drug concentrations used in the model were based on concentrations measured in target tissue in other studies(*77*), and we estimated what drug concentration was required in the media to achieve this concentration in the biopsy sample. Tissue drug concentration in the biopsy sample was not measures. Isotope labelling and immunofluorescence of the drug in the target tissue is something to consider in order to more accurately measure the drug concentrations in this model. The time of the incubation period was chosen for pragmatic reasons. Firstly, to avoid bacterial and fungal contamination of the tissue culture, and also as no known time period of incubation was known to be effective an arbitrary time point was chosen at 24 hours.

3.2.2 Patient data

	UC n=8	Colonic Crohn's n=8	Ileal Crohn's n=7	Normal n=8
Age	49 (27-76)	50 (20-75)	40 (20-66)	71 (42-86)
Sex M/F	4/4	5/3	6/1	5/3
Smoker	1	1	2	1
Disease length (months)	82 (17-160)	75 (0-169)	73 (0-169)	0 (0-0)
Mayo Score	9 (6-12)	NA	NA	1 (0-2)
Truelove and Witts	1 Mild 3 Moderate 4 Severe	NA	NA	6 Normal
CDAI	NA	238 (91-352)	221 (84-343)	15 (0-70)
Harvey Bradshaw Index	NA	10 (4-14)	9 (2-15)	0 (0-2)
Haemoglobin	118 (101-151)	111 (92-134)	112 (92-142)	127 (92-161)
CRP	62 (21-108)	54 (14-136)	63 (37-89)	13 (1-44)
Albumin	36 (29-42)	35 (31-41)	37 (32-44)	40 (30-50)

Table 25. Patient demographics, clinical scores and clinical indices. Active UC biopsies were defined by a Mayo score of 6 or greater. Active Crohn's was defined as a CDAI of greater than 150 or an HBI of 5 or greater

3.2.3 Ulcerative Colitis

3.2.3.1 microRNA expression can be altered in ulcerative colitis by drug treatment in an ex vivo model

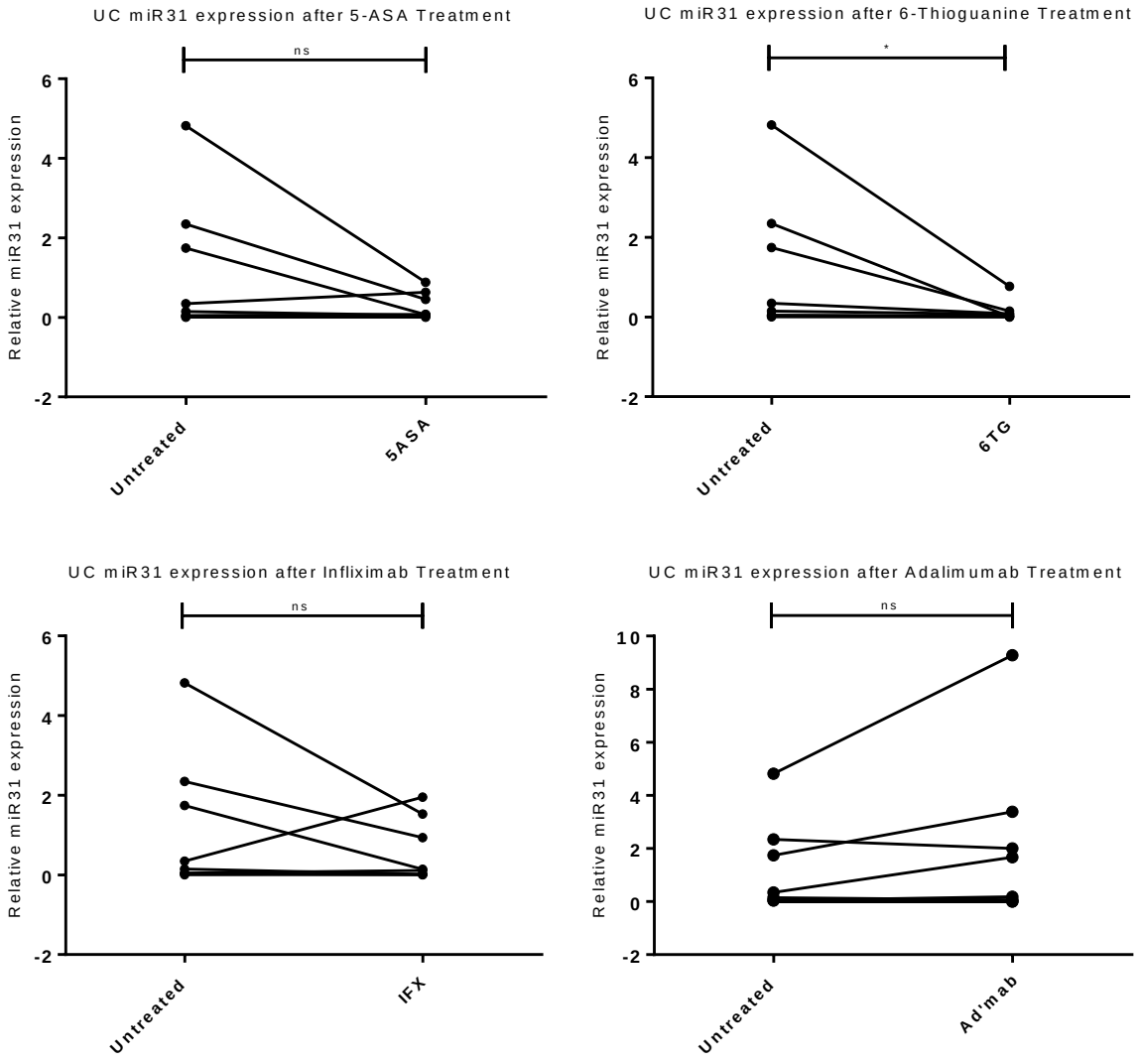


Figure 28. Ex vivo model showing the effects of treatment on miR-31 expression in active UC. n=8

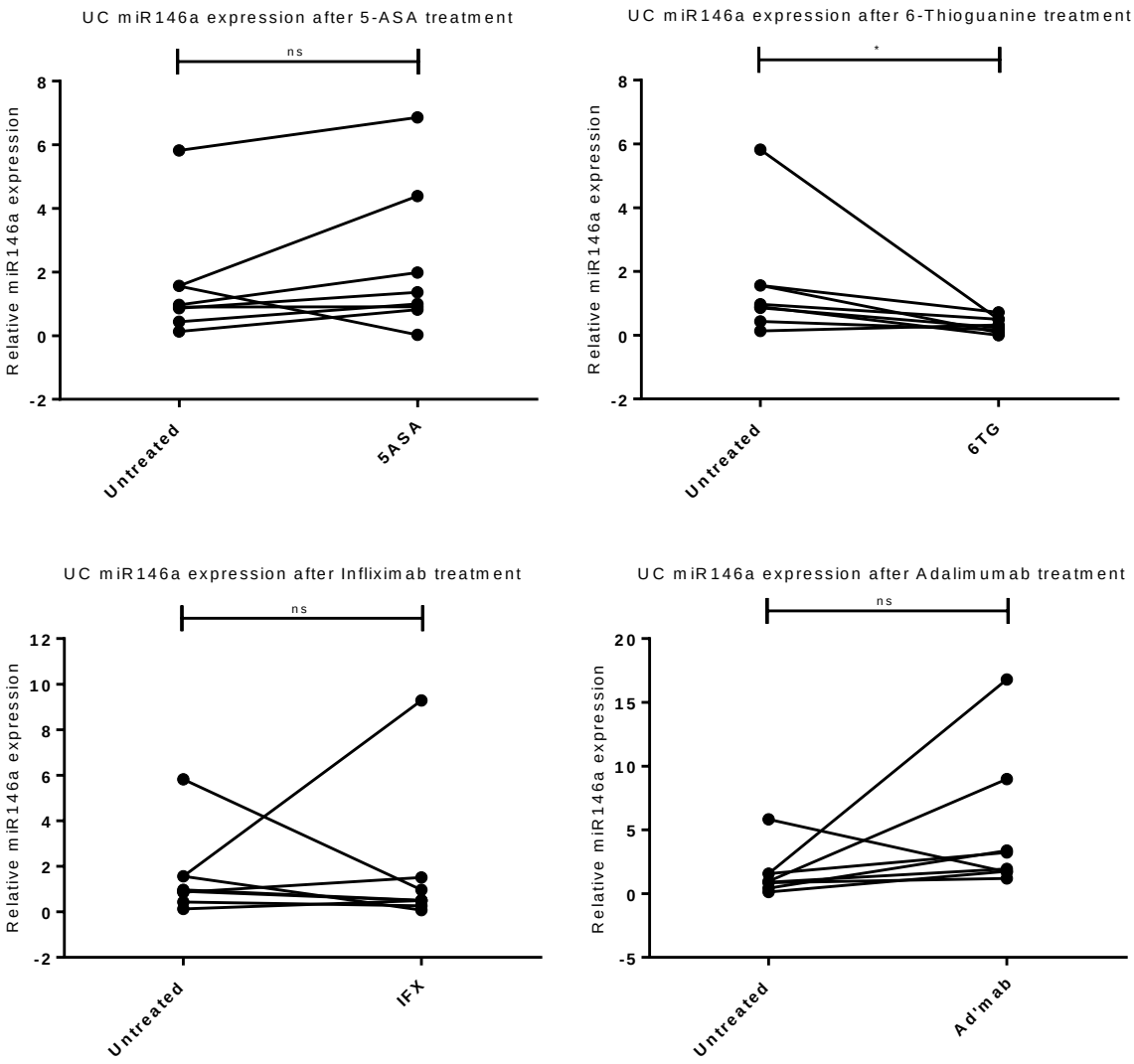


Figure 29. Ex vivo model showing the effects of treatment on miR-146a expression in active UC. n=8

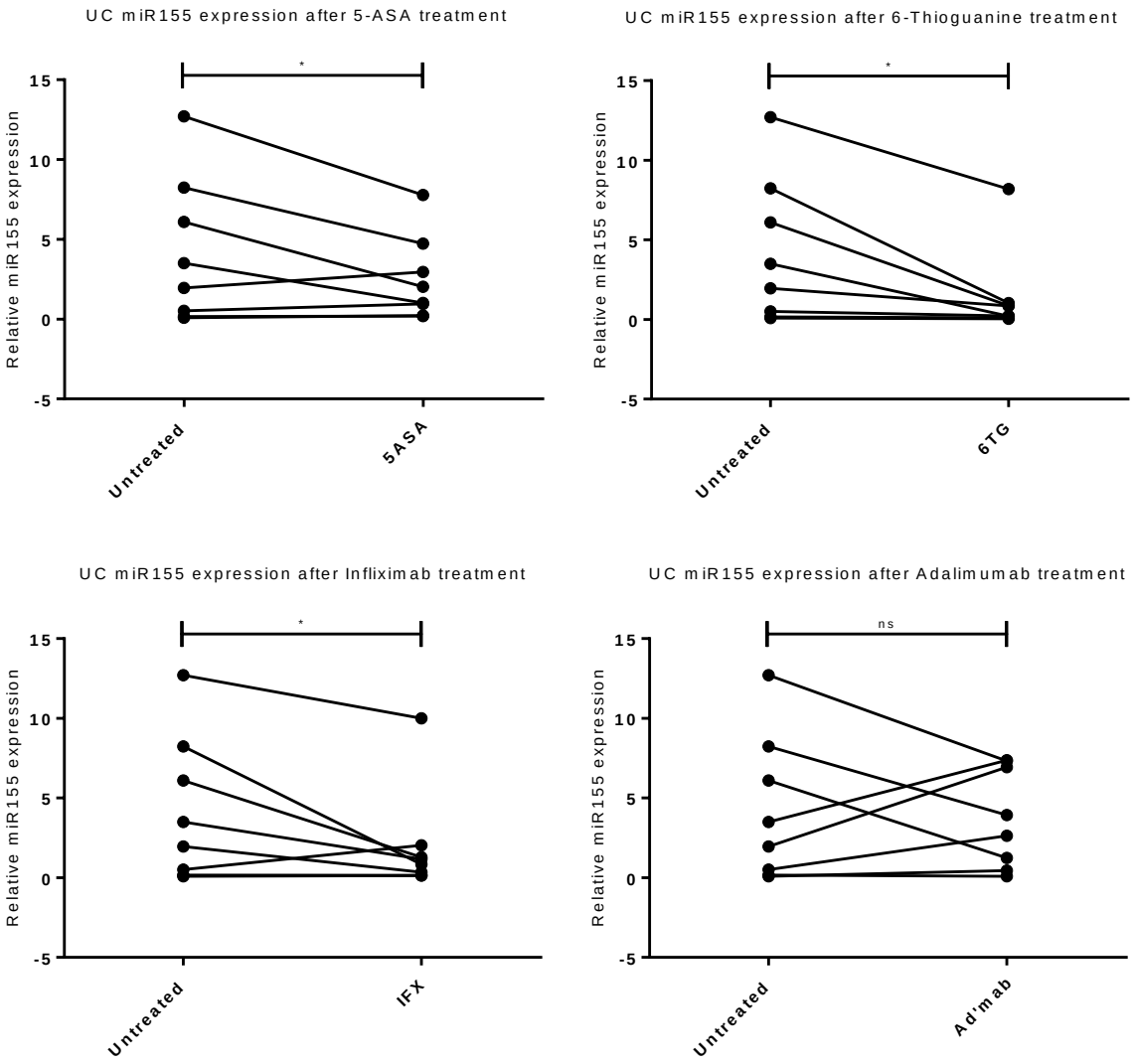


Figure 30. Ex vivo model showing the effects of treatment on miR-155 expression in active UC. n=8

The only treatment that significantly reduces miR-31 expression in active UC is 6-Thioguanine, although there is possibly a trend for infliximab (p=0.1279) and 5-ASA (p=0.1259) to do the same. As seen in the in vivo study Adalimumab does not reduce miR-31 expression.

When active treatment naïve UC is treated miR-146a expression remains unchanged in all the treatment groups except with the treatment of 6-Thioguanine, where the miR-146a expression is reduced after treatment, as seen in the in vivo study.

miR-155 expression in active UC is significantly reduced by the treatment of 5-ASA, 6-Thioguanine, and infliximab, but this effect is not seen with Adalimumab treatment. These effects are modest compared to the in vivo study but nevertheless consistent.

3.2.3.2 Cytokine expression can be altered in ulcerative colitis by drug treatment in an ex vivo model

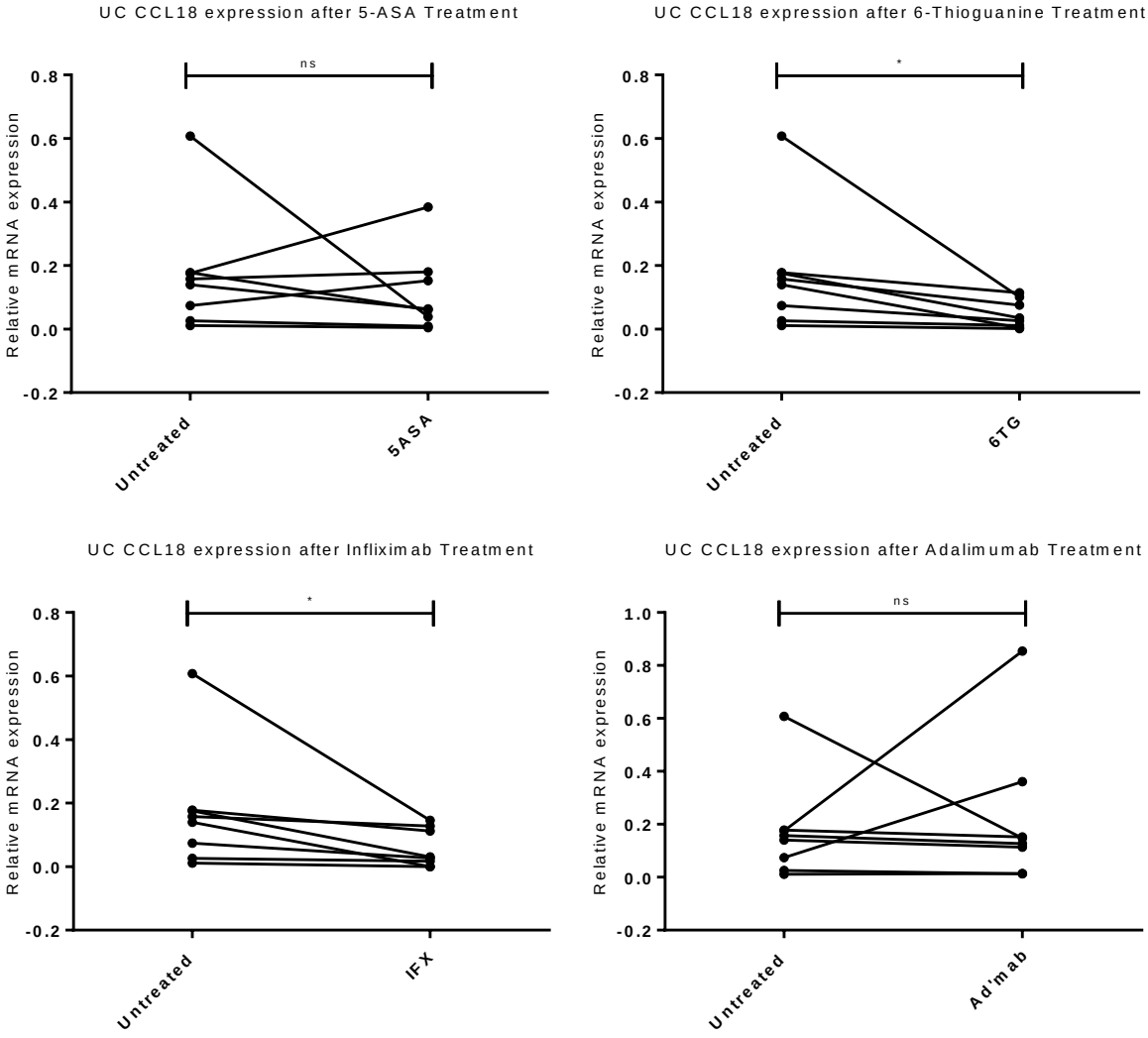


Figure 31. Ex vivo model showing the effects of treatment on CCL18 expression in active UC. n=8

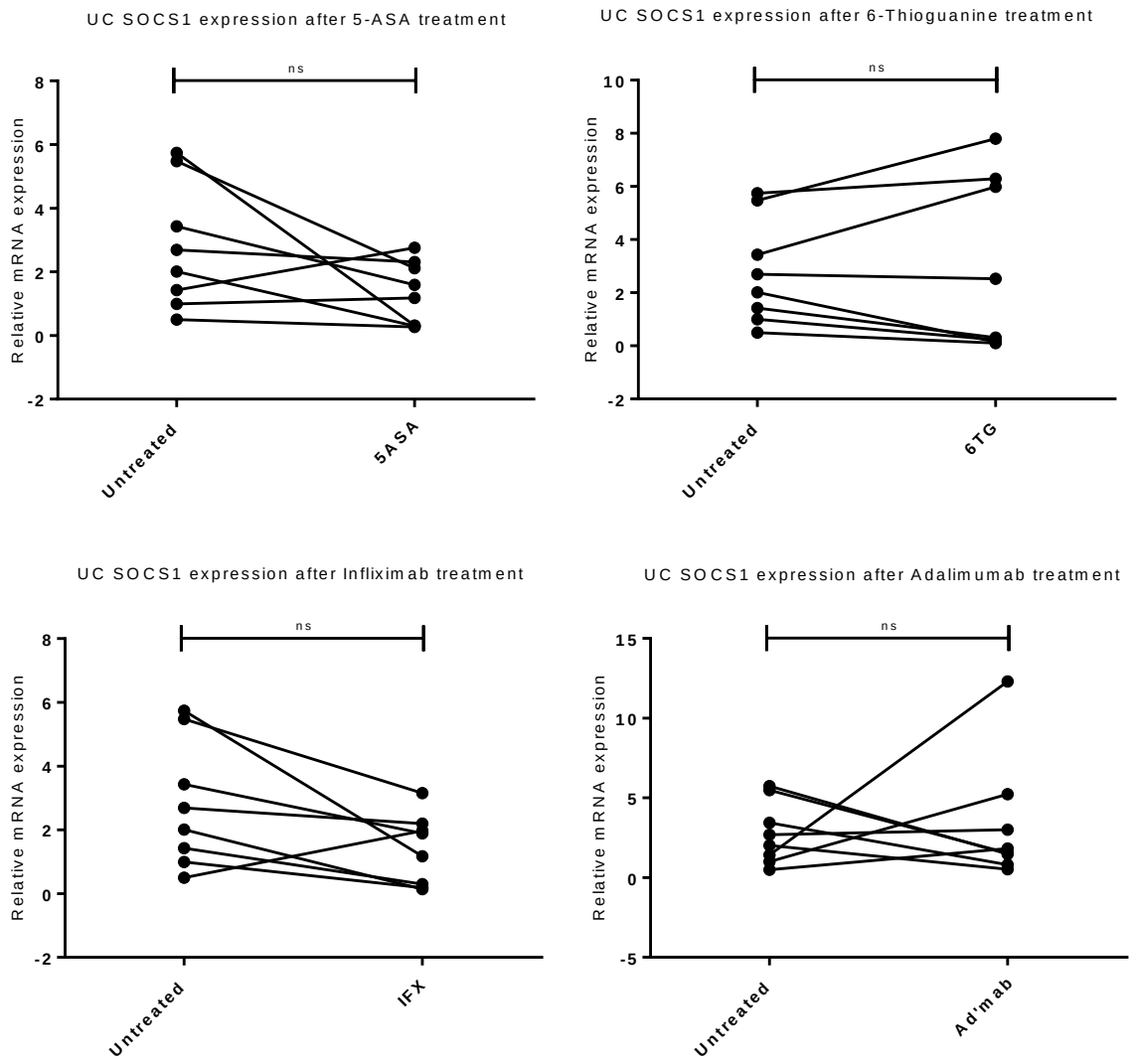


Figure 32. Ex vivo model showing the effects of treatment on SOCS1 expression in active UC. n=8

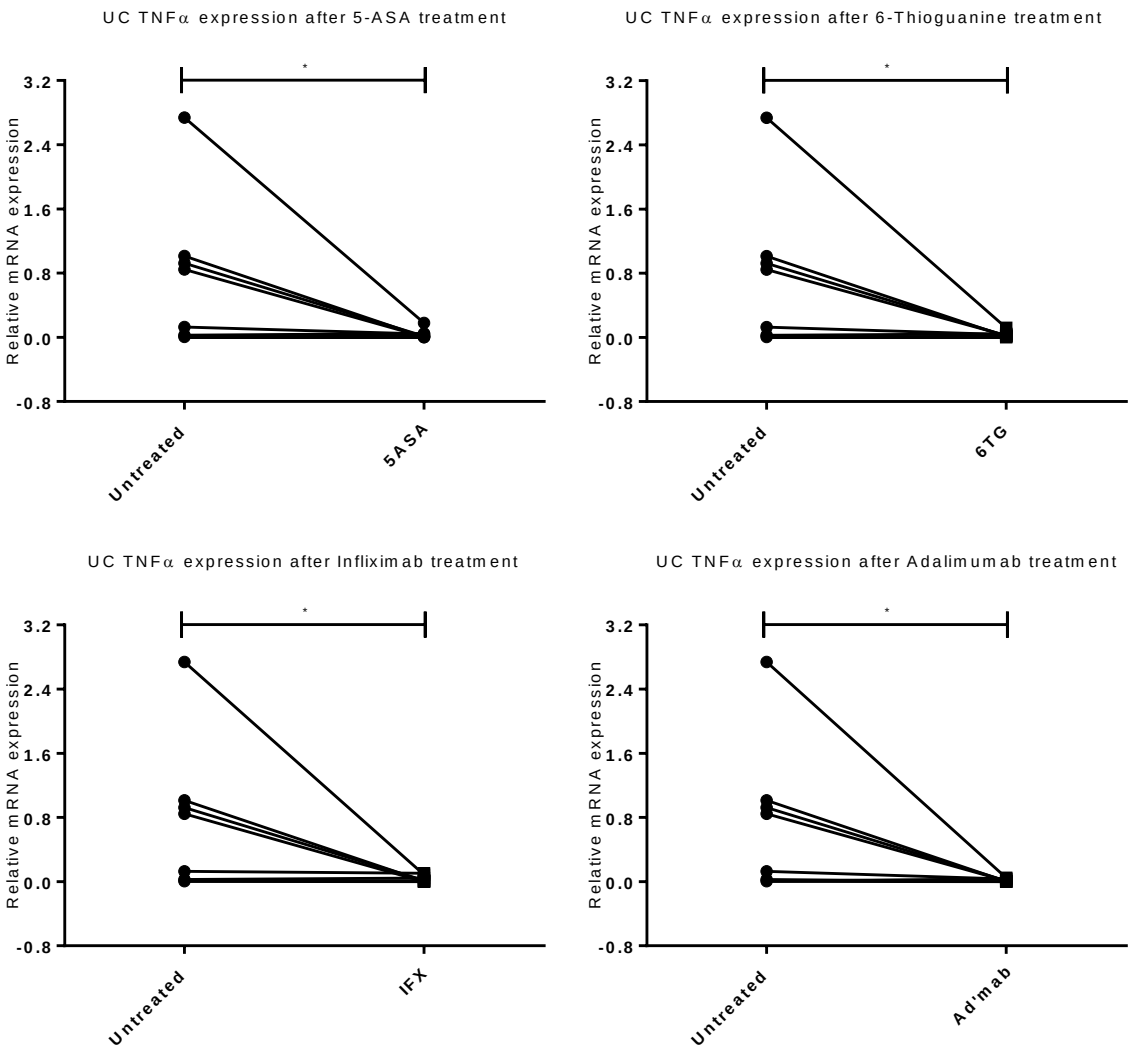


Figure 33. Ex vivo model showing the effects of treatment on TNFα expression in active UC. n=8

CCL18 expression in active UC does reduce as a result of 6-Thioguanine and Infliximab treatment, as seen in the in vivo study. However, 5-ASA and Adalimumab therapy does not have the same effect as we had seen in the in vivo study, with no significant difference in CCL18 expression before and after treatment.

There is no difference in the expression of SOCS1 between treatment naïve active UC, before and after treatment with 5-ASA, 6-Thioguanine, Infliximab, or Adalimumab.

As we saw in the in vivo study with unpaired samples, TNFα expression is significantly reduced in active UC when treated with any of the therapies used in this study.

3.2.3.3 DMSO Control Treatments

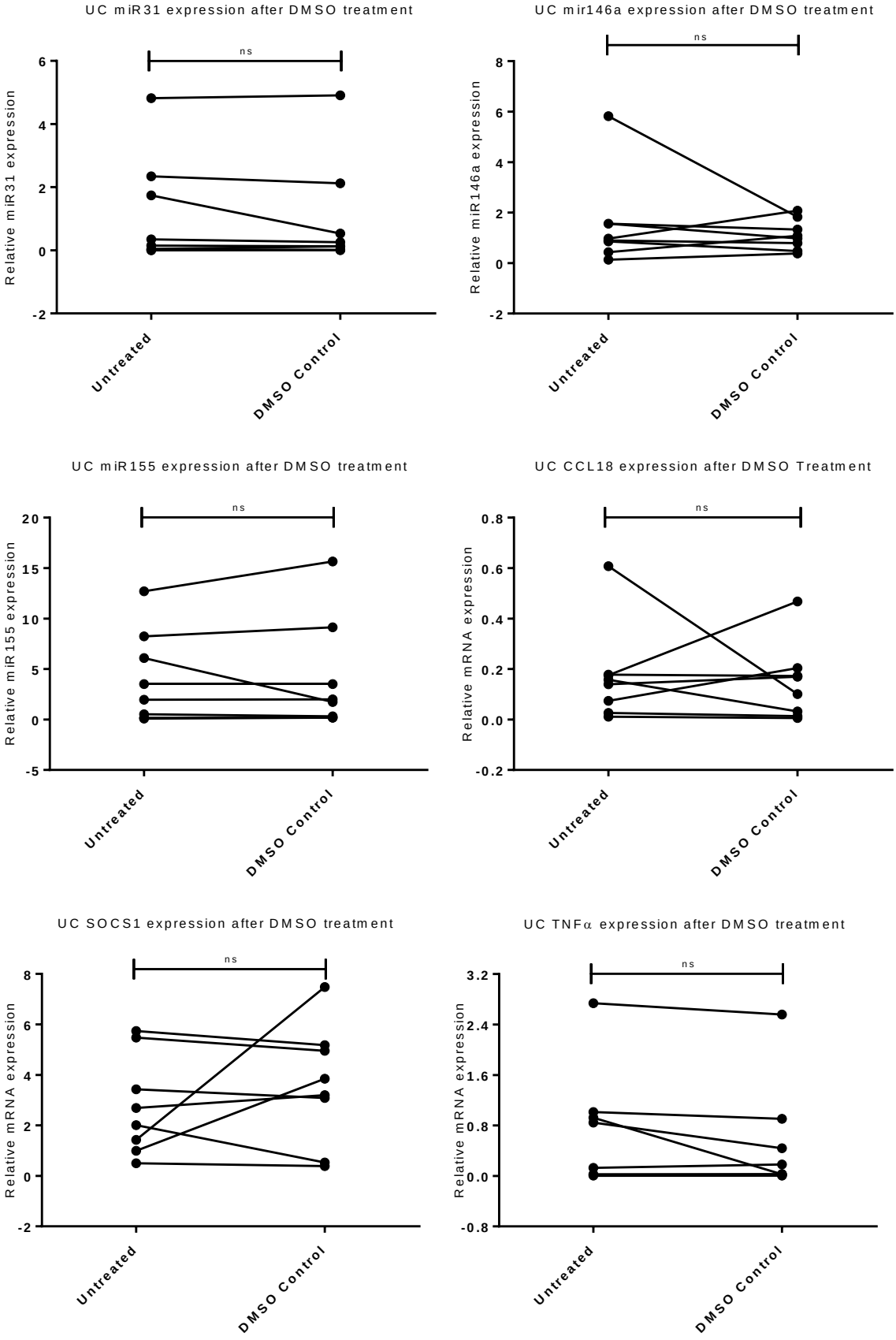


Figure 34. Ex vivo model showing the effects of DMSO on expression in active UC. n=8

3.2.4 Colonic Crohn's Disease

3.2.4.1 microRNA expression can be altered in Crohn's colitis by drug treatment in an ex vivo model

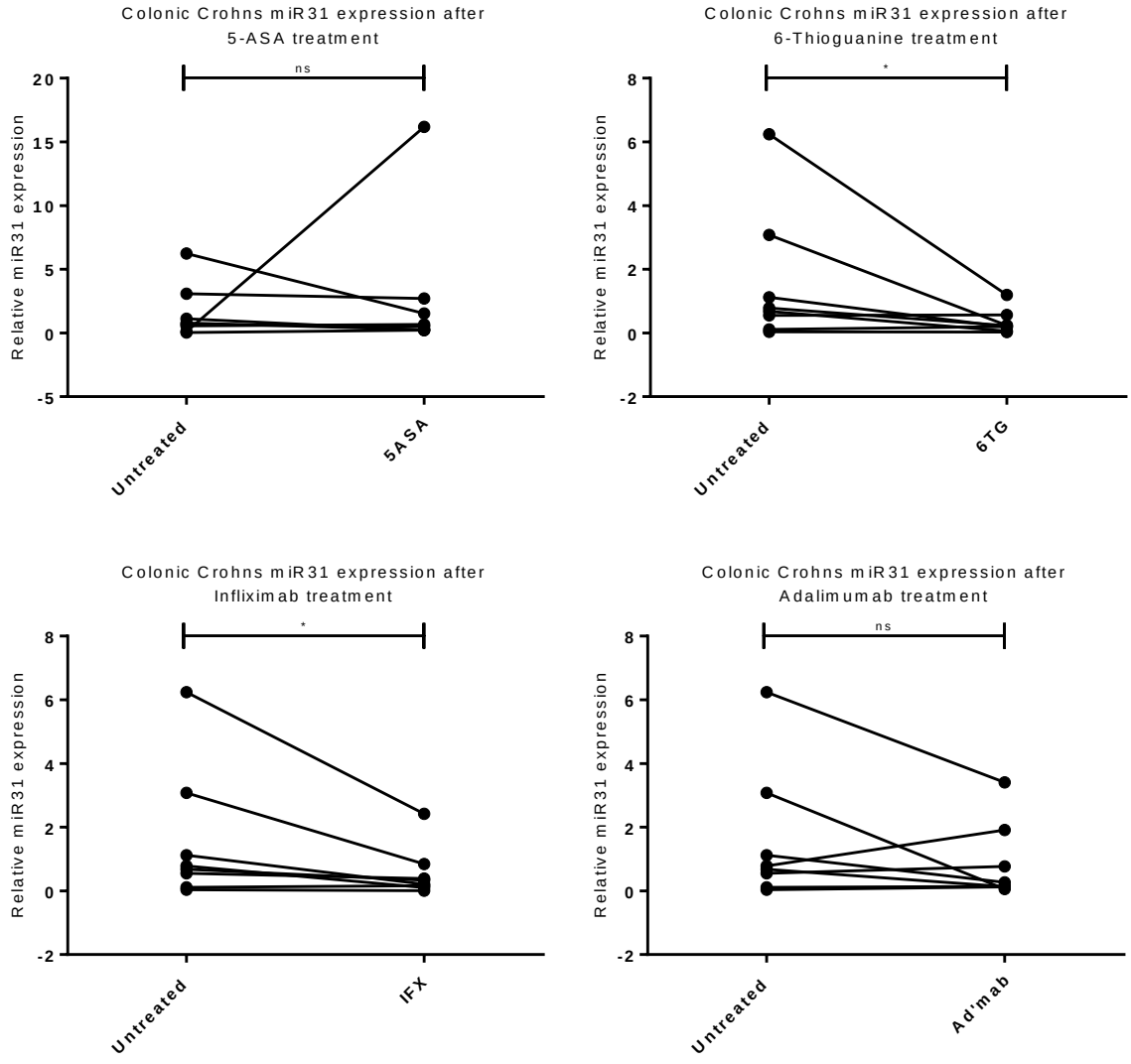


Figure 35. Ex vivo model showing the effects of treatment on miR-31 expression in active colonic Crohn's Disease. n=8

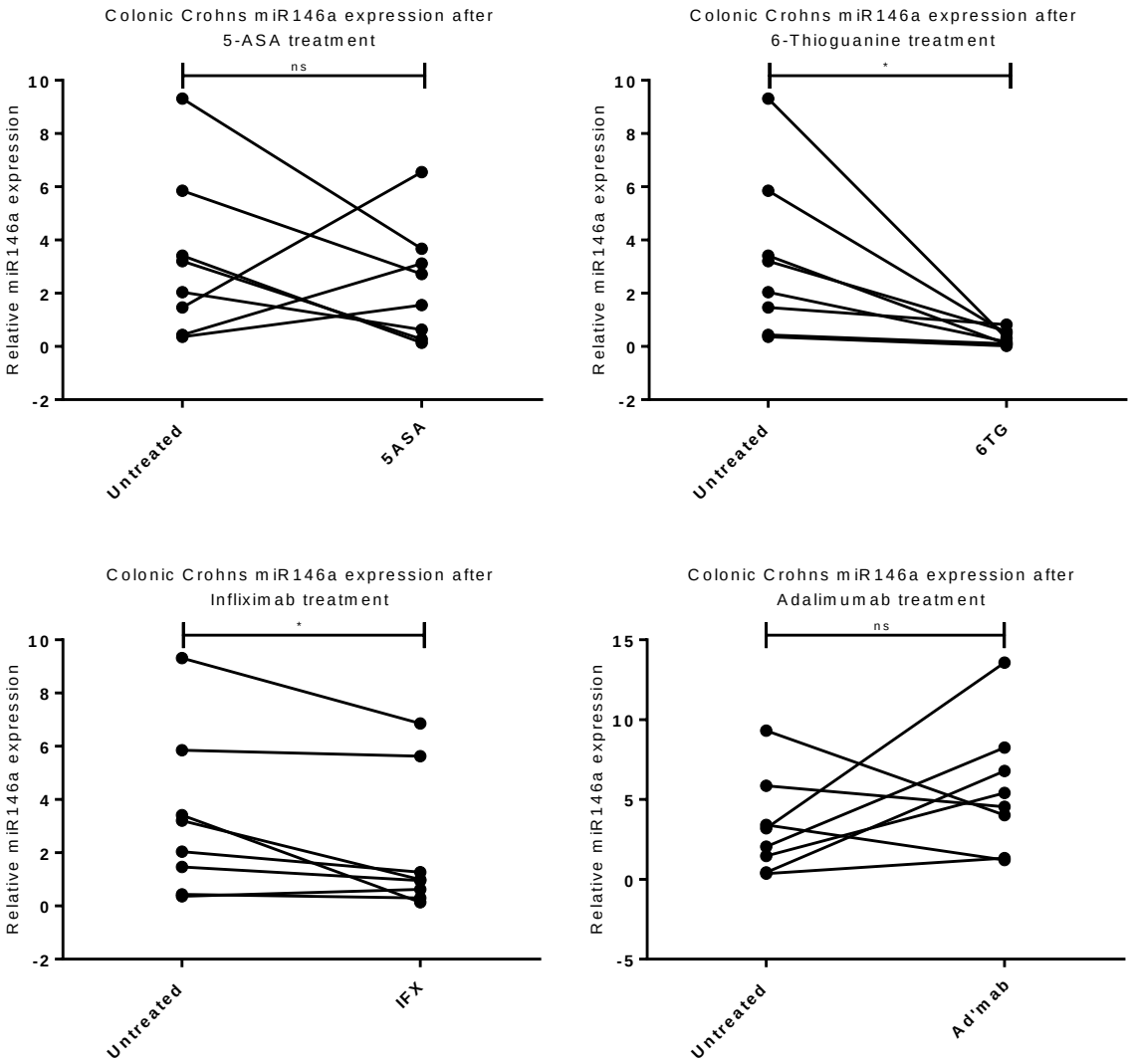


Figure 36. Ex vivo model showing the effects of treatment on miR-146a expression in active colonic Crohn's Disease. n=8

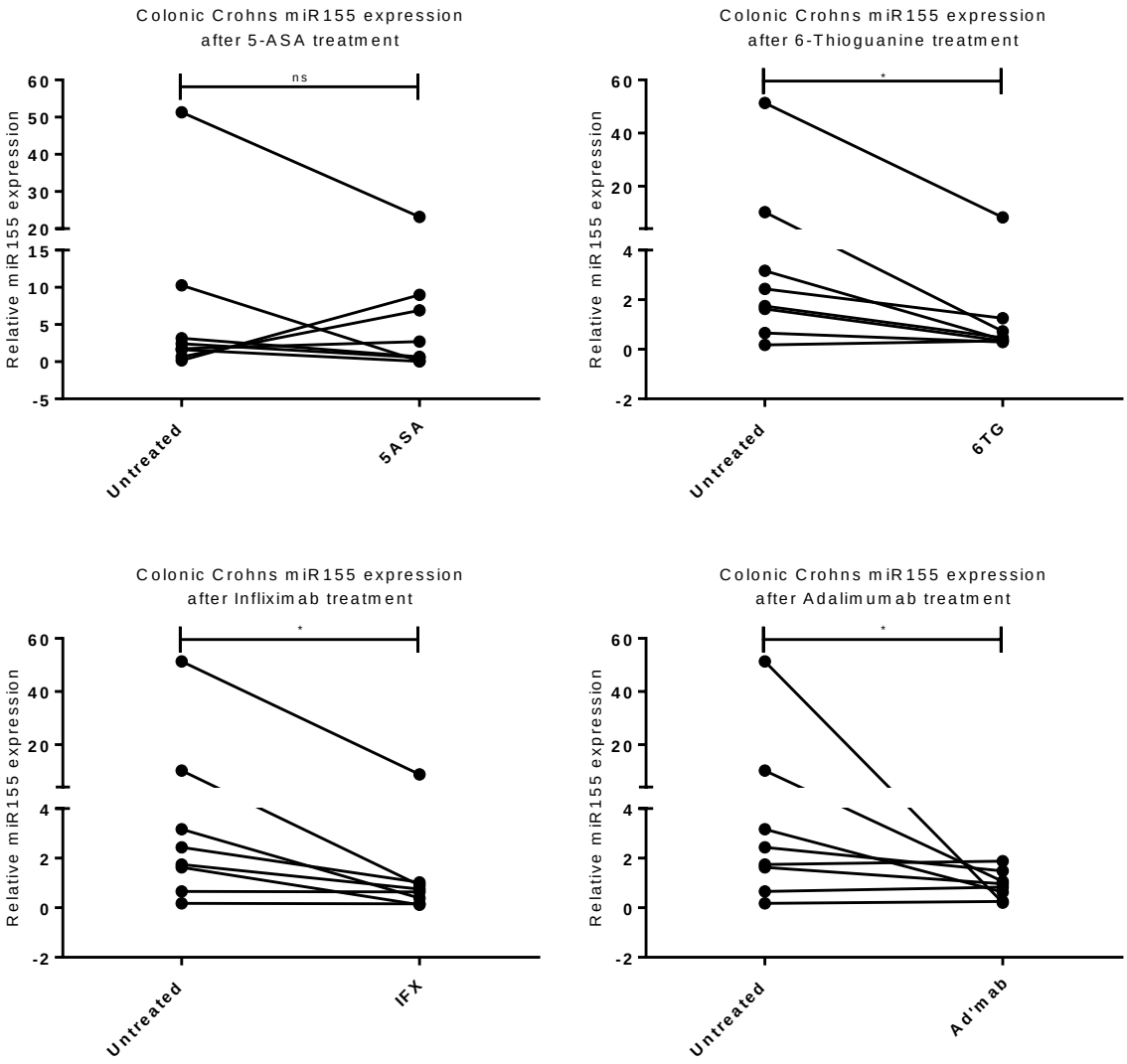


Figure 37. Ex vivo model showing the effects of treatment on miR-155 expression in active colonic Crohn's Disease. n=8

As was seen in the in vivo study, miR-31 expression is down regulated by 6-Thioguanine in active sigmoid Crohn's colitis, but not by 5-ASA. However here miR-31 expression is also significantly reduced by infliximab treatment as well and there is a trend for Adalimumab to do the same, which was not seen in the in vivo study with unpaired groups.

In active Crohn's colitis miR-146a expression can be reduced using a Thiopurine as seen in the in vivo study, and confirmed here with the culture of colonic biopsies with active Crohn's with 6-Thioguanine. However, miR-146a expression is also reduced here by infliximab that was not seen in the in vivo experiment. Also, the 5-ASA treatment has not significantly reduced miR-146a expression in this model, which was seen in the unpaired samples.

5-ASA therapy is the only treatment that does not appear to reduce miR-155 expression in active Crohn's colitis. But similarly, to UC, anti-TNF treatment and 6-Thioguanine does reduce miR-155 expression in active Crohn's colitis. In the in vivo study Infliximab did not significant reduce miR-155 expression, however the patient groups there were different so confounding factors may be at work.

3.2.4.2 Cytokine expression can be altered in Crohn's colitis by drug treatment in an ex vivo model

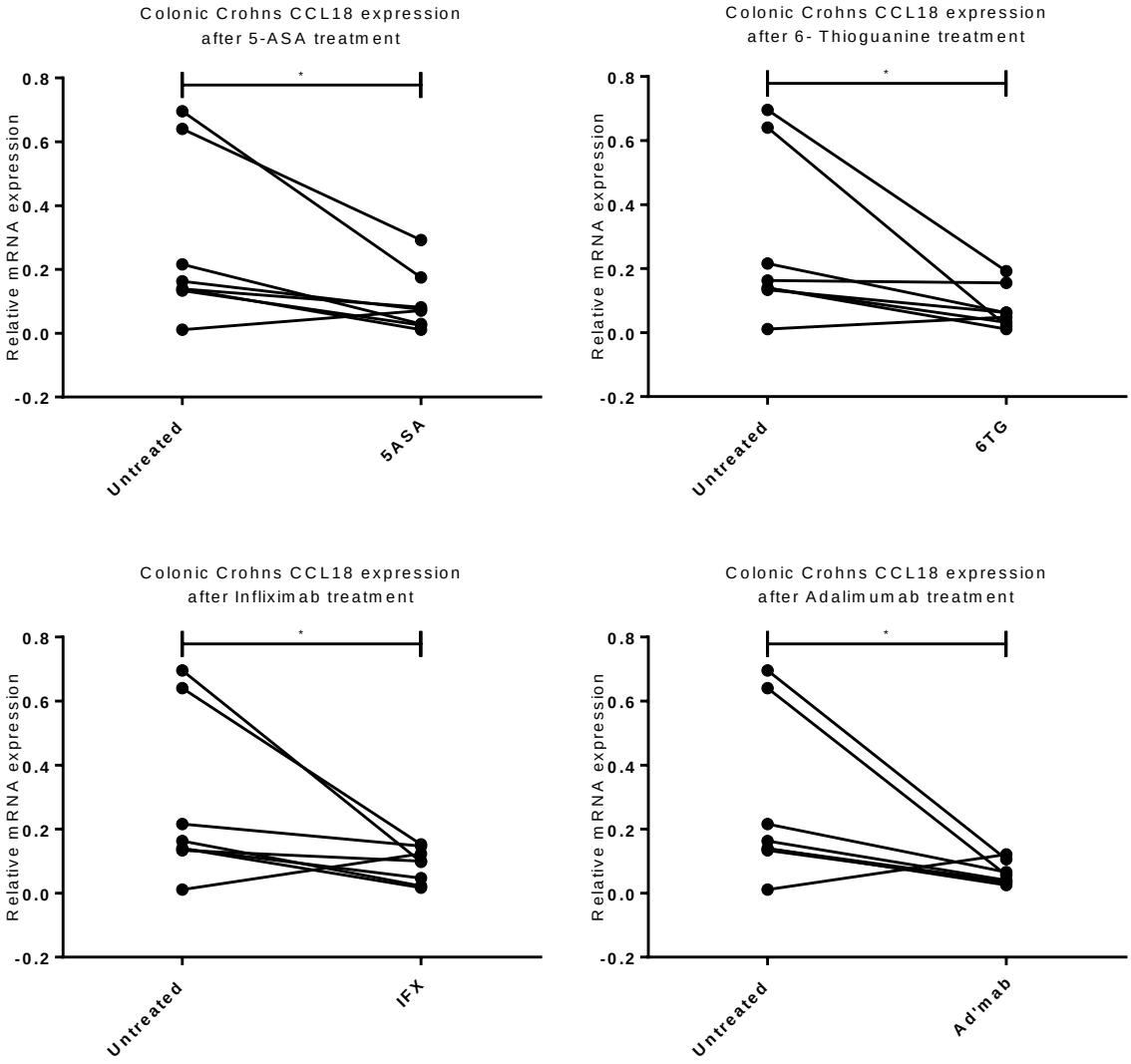


Figure 38. Ex vivo model showing the effects of treatment on CCL18 expression in active colonic Crohn's Disease. n=8

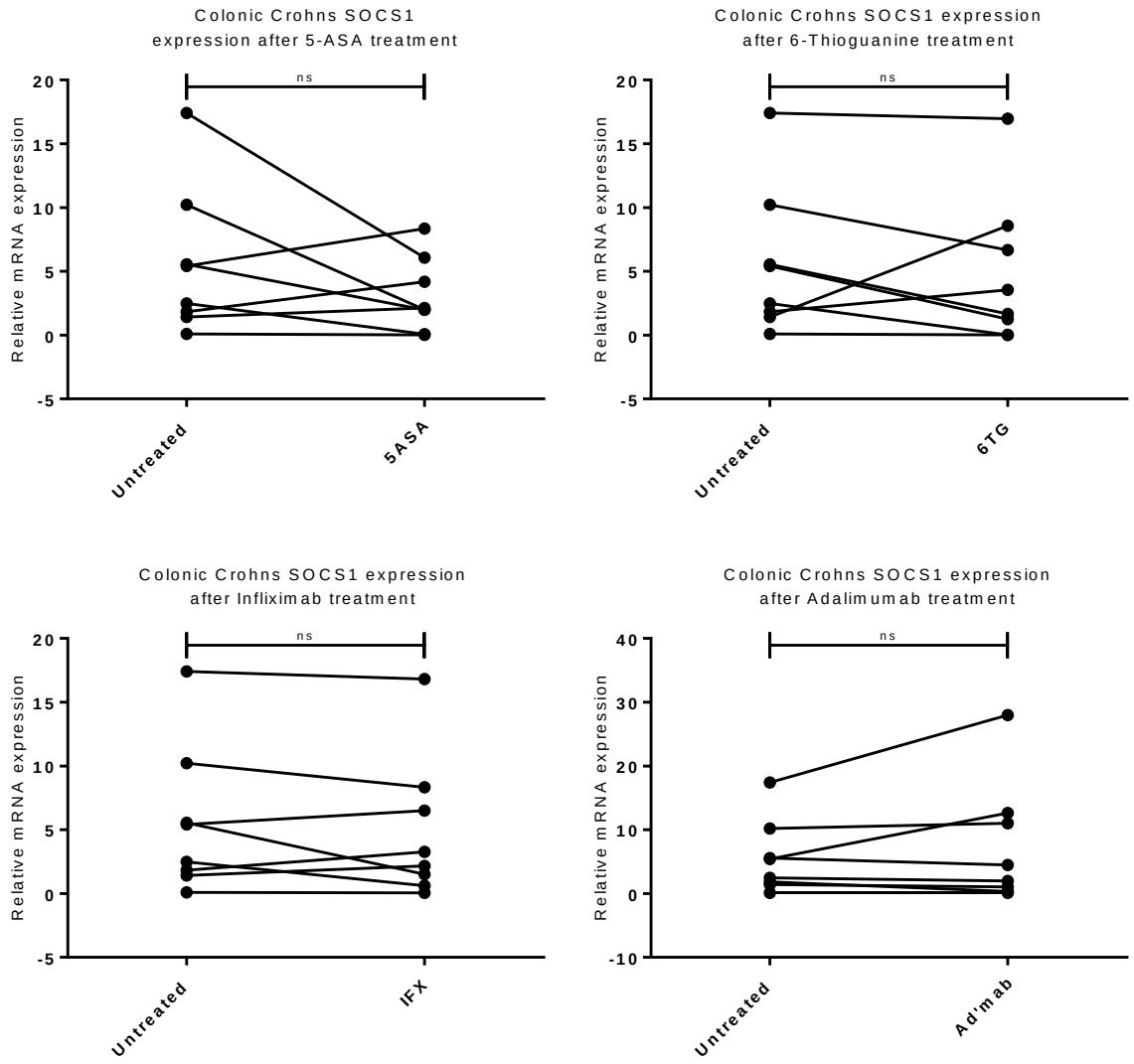


Figure 39. Ex vivo model showing the effects of treatment on SOCS1 expression in active colonic Crohn's Disease. n=8

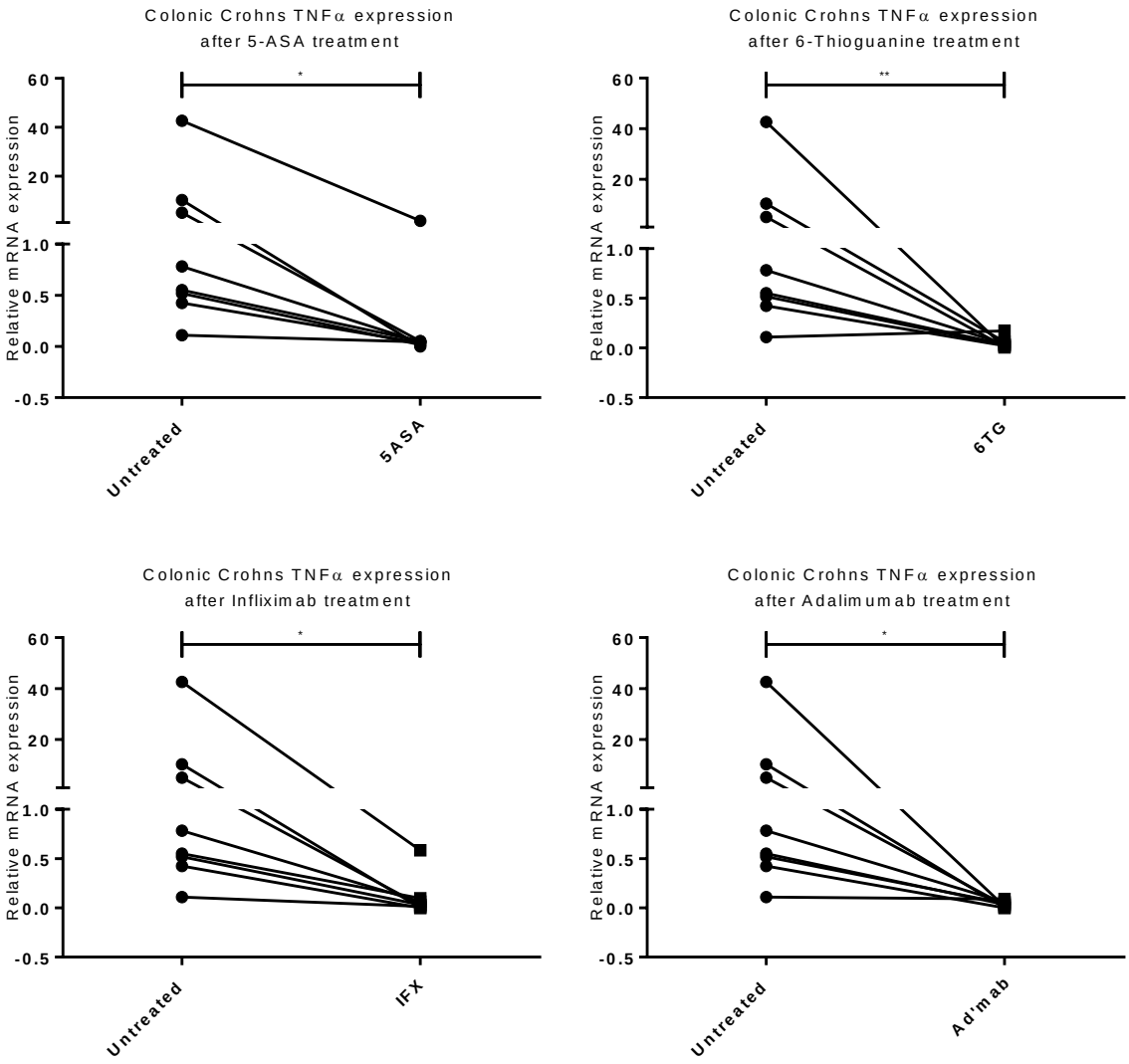


Figure 40. Ex vivo model showing the effects of treatment on TNFα expression in active colonic Crohn's Disease. n=8

As was seen in the in vivo study, CCL18 treatment of active Crohn's colonic biopsies with all four of the therapies is reduced. Also, similarly to the in vivo study this effect treatment has on reducing CCL18 expression in active Crohn's colitis is more profound than the effect seen in UC.

There is no difference in the expression of SOCS1 between treatment naïve active Crohn's colitis, before and after treatment with 5-ASA, 6-Thioguanine, Infliximab, or Adalimumab. This is also seen in the UC samples and in the in vivo study of patients with Crohn's colitis.

As was seen in both the in vivo study of Crohn's colitis and the ex vivo study with UC, there is a profound reduction in TNFα expression following the treatment of active treatment naïve Crohn's colitis with all the therapies.

3.2.4.3 DMSO Control Treatments

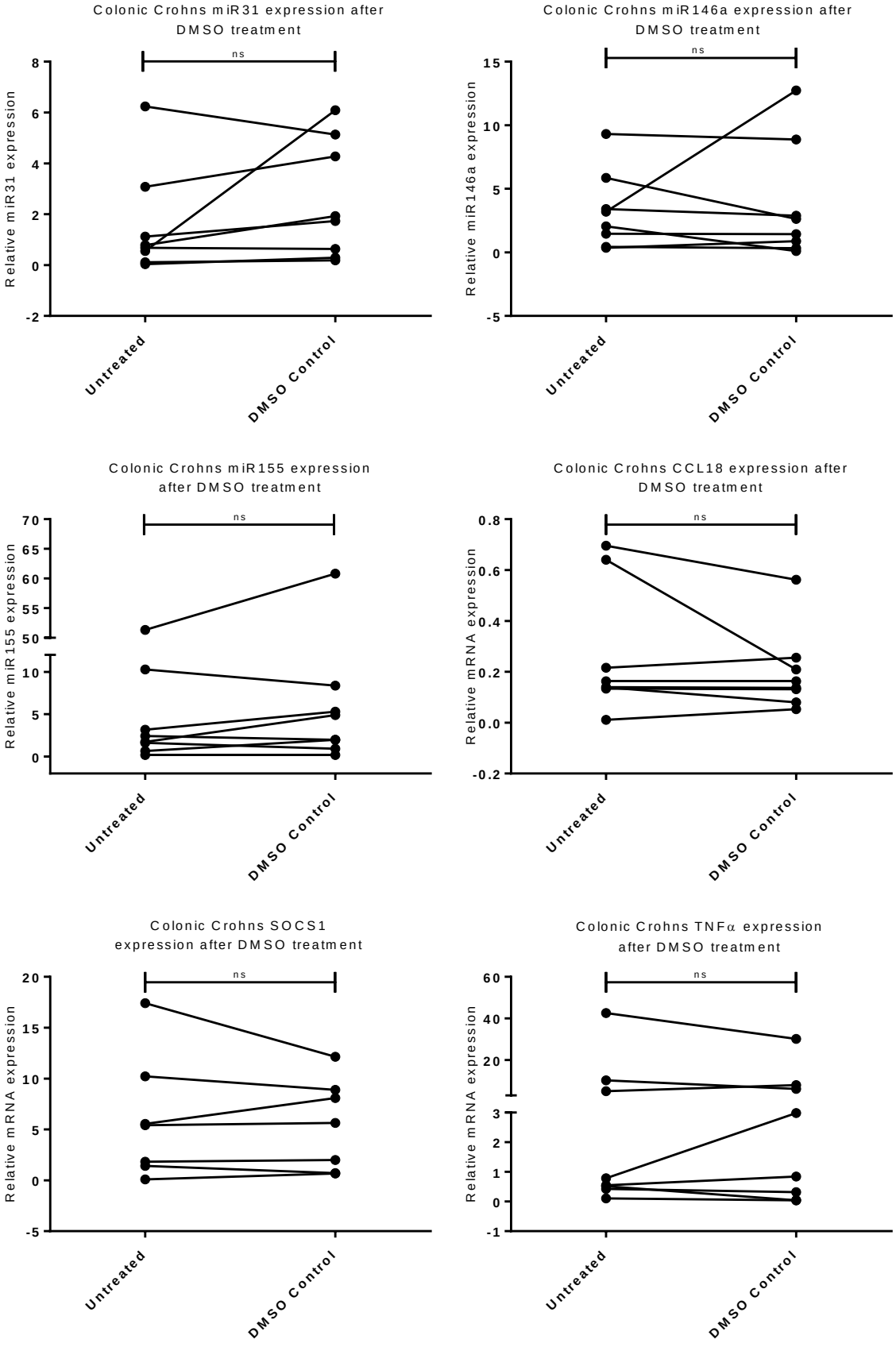


Figure 41. Ex vivo model showing the effects of DMSO on expression in active colonic Crohn's Disease. n=8

3.2.5 Ileal Crohn's Disease

3.2.5.1 microRNA expression can be altered in Crohn's ileitis by drug treatment in an ex vivo model

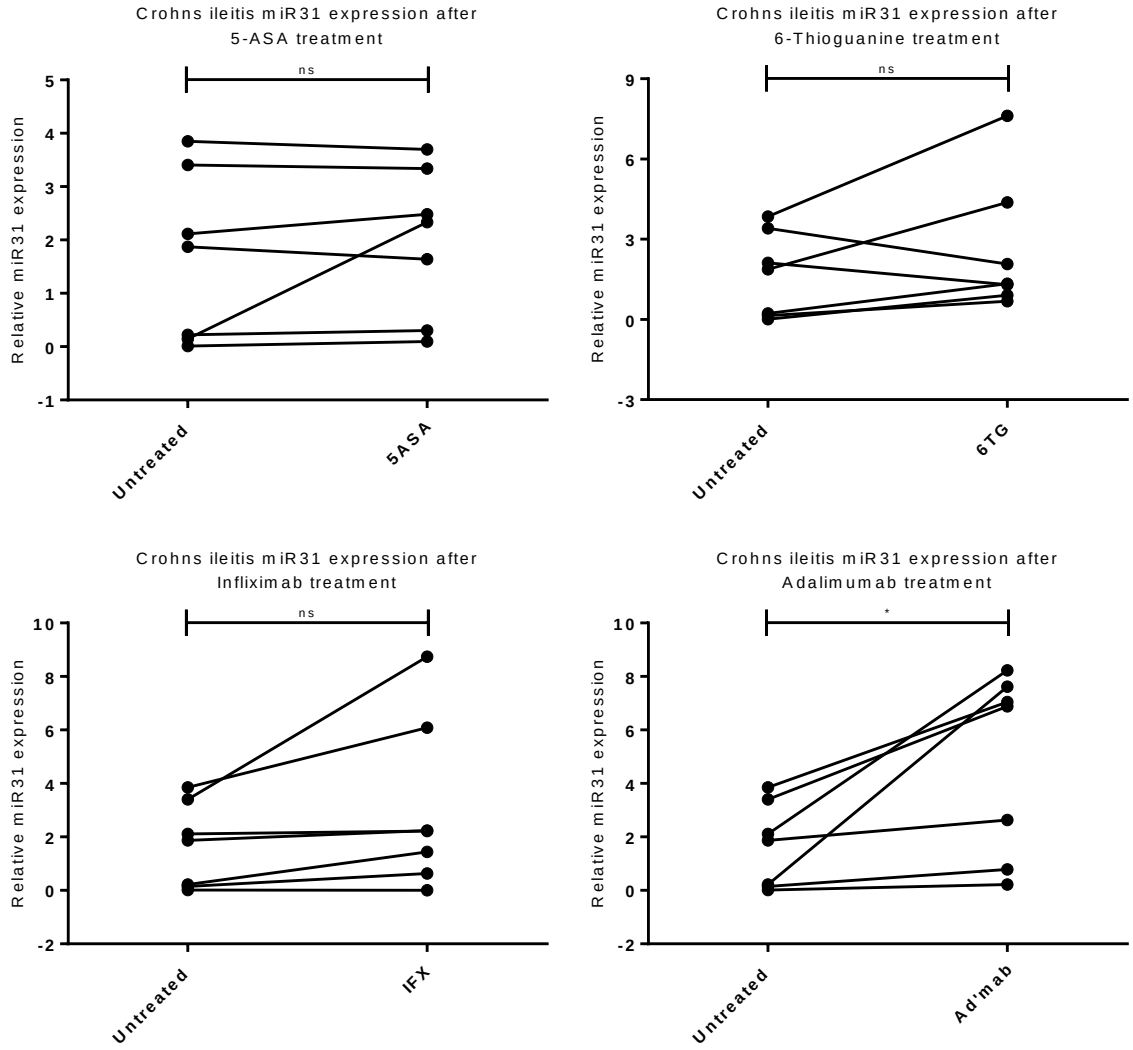


Figure 42. Ex vivo model showing the effects of treatment on miR-31 expression in active Crohn's Ileitis. n=7

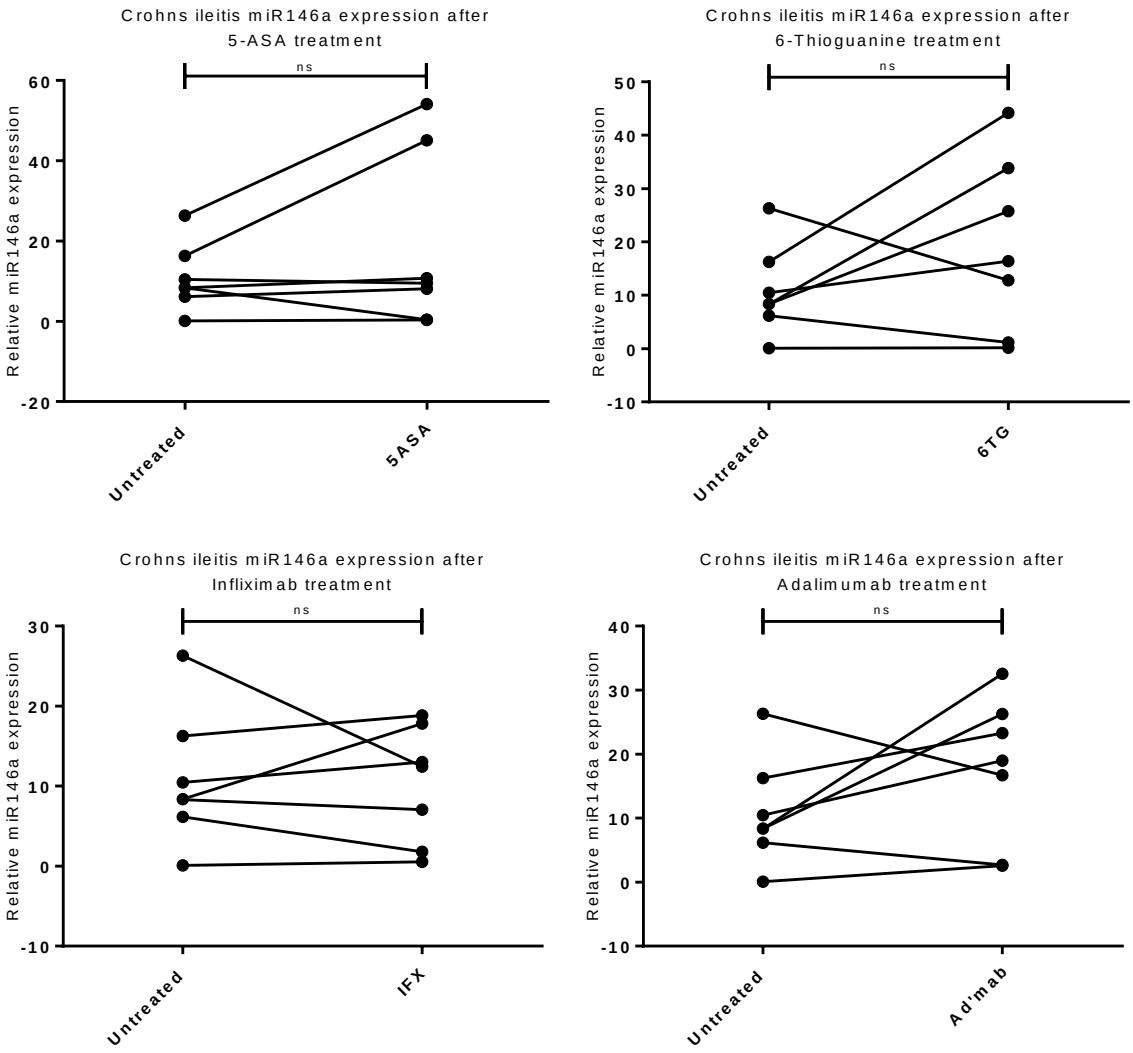


Figure 43. Ex vivo model showing the effects of treatment on miR-146a expression in active Crohn's Ileitis. n=7

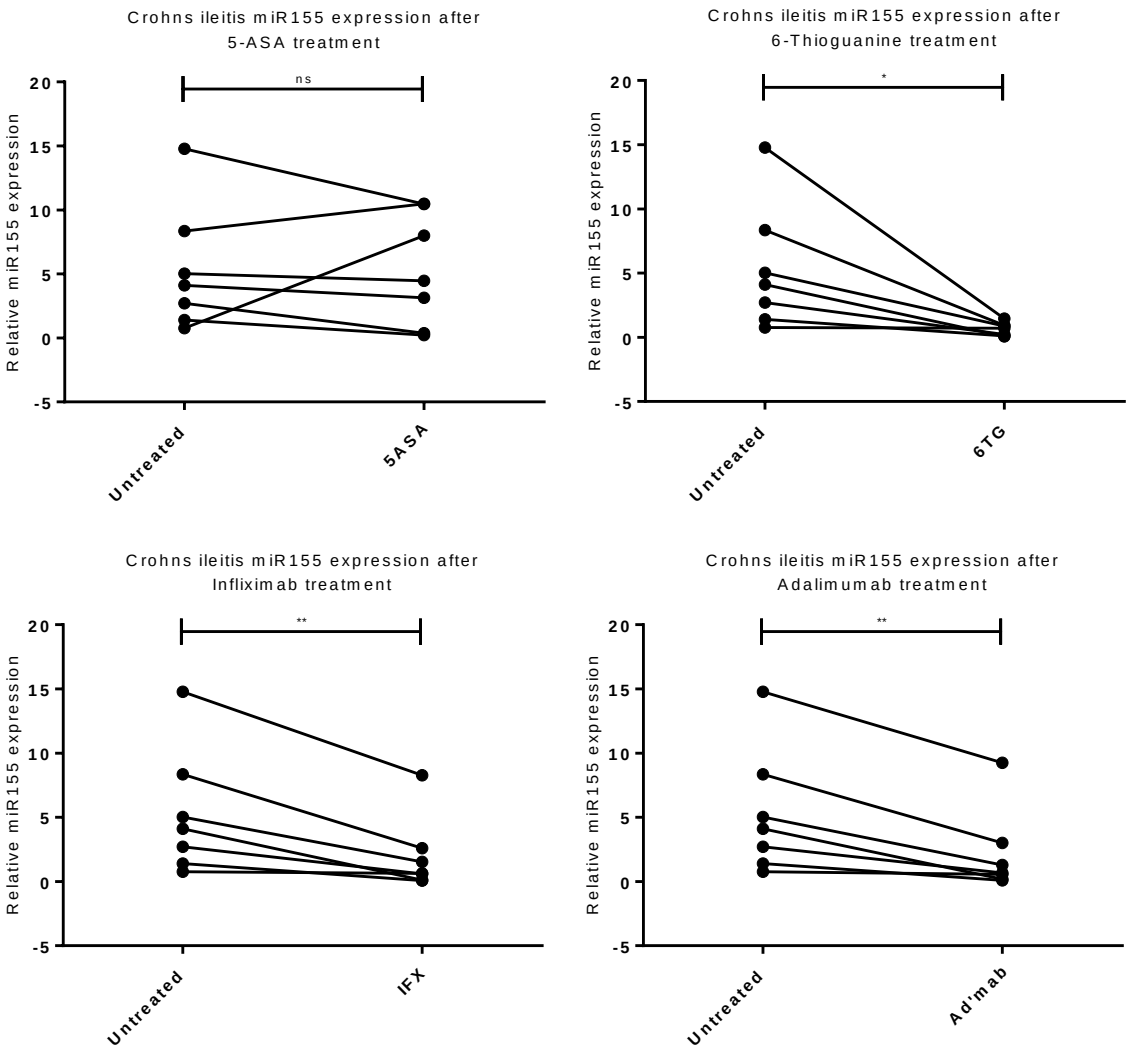


Figure 44. Ex vivo model showing the effects of treatment on miR-155 expression in active Crohn's Ileitis. n=7

In the in vivo study a non-significant trend for increased expression of miR-31 was seen in the ileal mucosa of Crohn's disease that had responded clinically and endoscopically to Azathioprine and Adalimumab. In the ex vivo experiment this trend is conserved with 6-Thiopurine treatment, and has shown significance with Adalimumab treatment. A trend for increasing miR-31 expression using Infliximab is also seen. 5-ASA does not seem to affect the miR-31 expression in the ileum of active Crohn's disease, which may in part explain its lack of clinical effect in small bowel inflammatory bowel disease.

As seen in the previous chapter, there is no significant difference in miR-146a expression after treatment of active ileal Crohn's disease. The trend of increased expression with Azathioprine and Adalimumab treatments is not as clearly identified here.

miR-155 expression is significantly reduced by anti-TNF therapy and 6-Thioguanine therapy in active ileal Crohn's disease. This pattern was also seen in the in vivo study. 5-ASA treatment, again, did not have this reducing effect in ileal mucosa, which again, may explain its lack of clinical effect in small bowel inflammatory bowel disease.

3.2.5.2 Cytokine expression can be altered in Crohn's ileitis by drug treatment in an ex vivo model

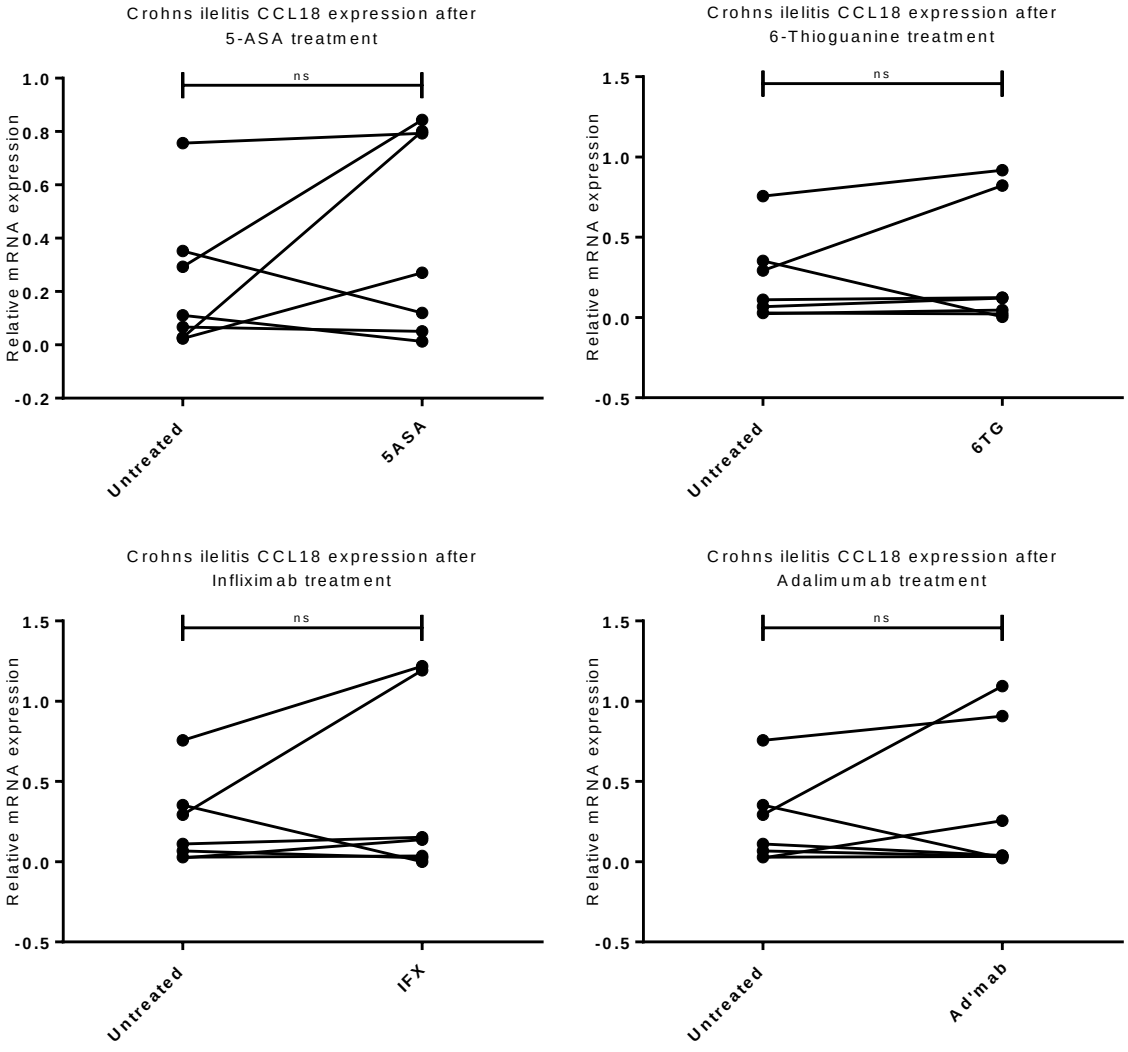


Figure 45. Ex vivo model showing the effects of treatment on CCL18 expression in active Crohn's Ileitis. n=7

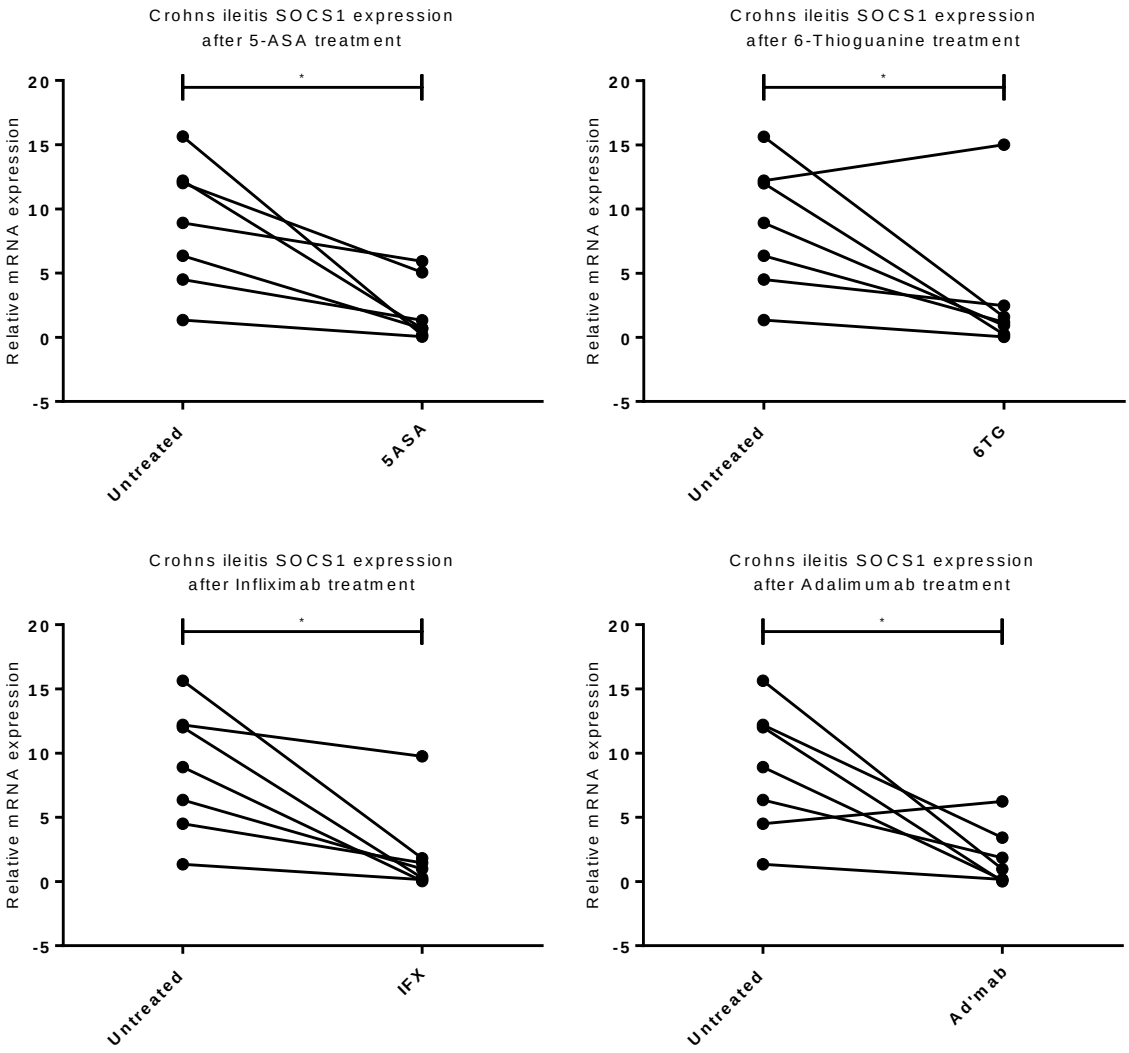


Figure 46. Ex vivo model showing the effects of treatment on SOCS1 expression in active Crohn's Ileitis. n=7

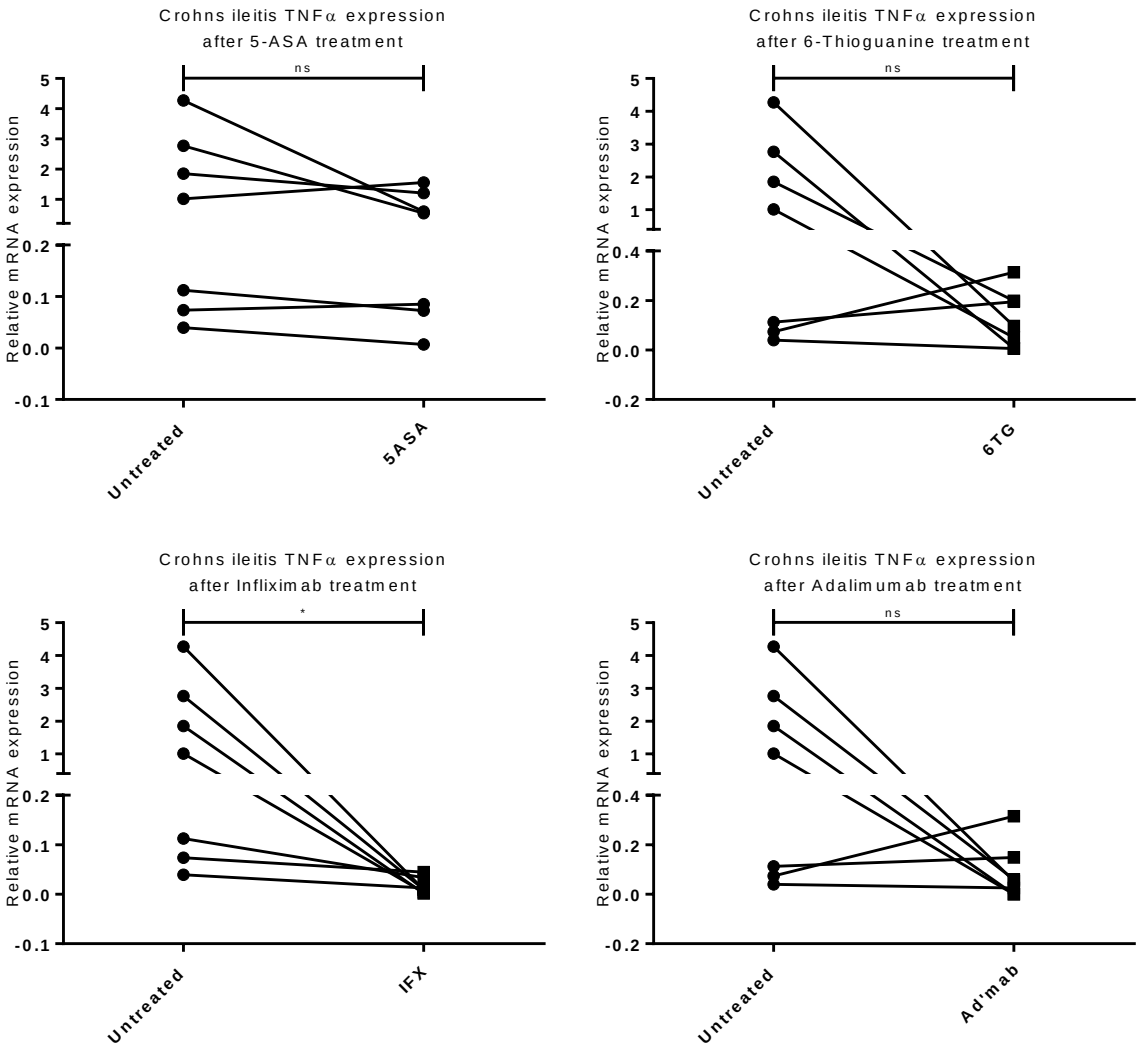


Figure 47. Ex vivo model showing the effects of treatment on TNFα expression in active Crohn's Ileitis. n=7

In the in vivo study reduced CCL18 expression was seen in the ileal mucosa of active Crohn's ileitis when compared to normal mucosa. However, the only treatment which increased the CCL18 expression significantly was Adalimumab. Here none of the treatment show significant increase CCL18 expression.

There was no significant change in SOCS1 expression in the in vivo study when looking at ileal Crohn's disease. However, SOCS1 expression in this experiment is decreased significantly following the treatment using all the therapies including 5-ASA. This suggests a differential expression between ileal inflammation and colonic inflammation. SOCS1 therefore may play a role in the pathogenesis of ileal Crohn's disease alone.

TNFα expression in active Crohn's Ileitis is significantly reduced by using infliximab treatment, as you might expect. This level of significance has not been met by 6-Thioguanine or Adalimumab, however there does appear to be a trend for TNFα expression to be reduced by these treatments. The trend is not so clearly seen with 5-ASA therapy.

3.2.5.3 DMSO Control Treatments

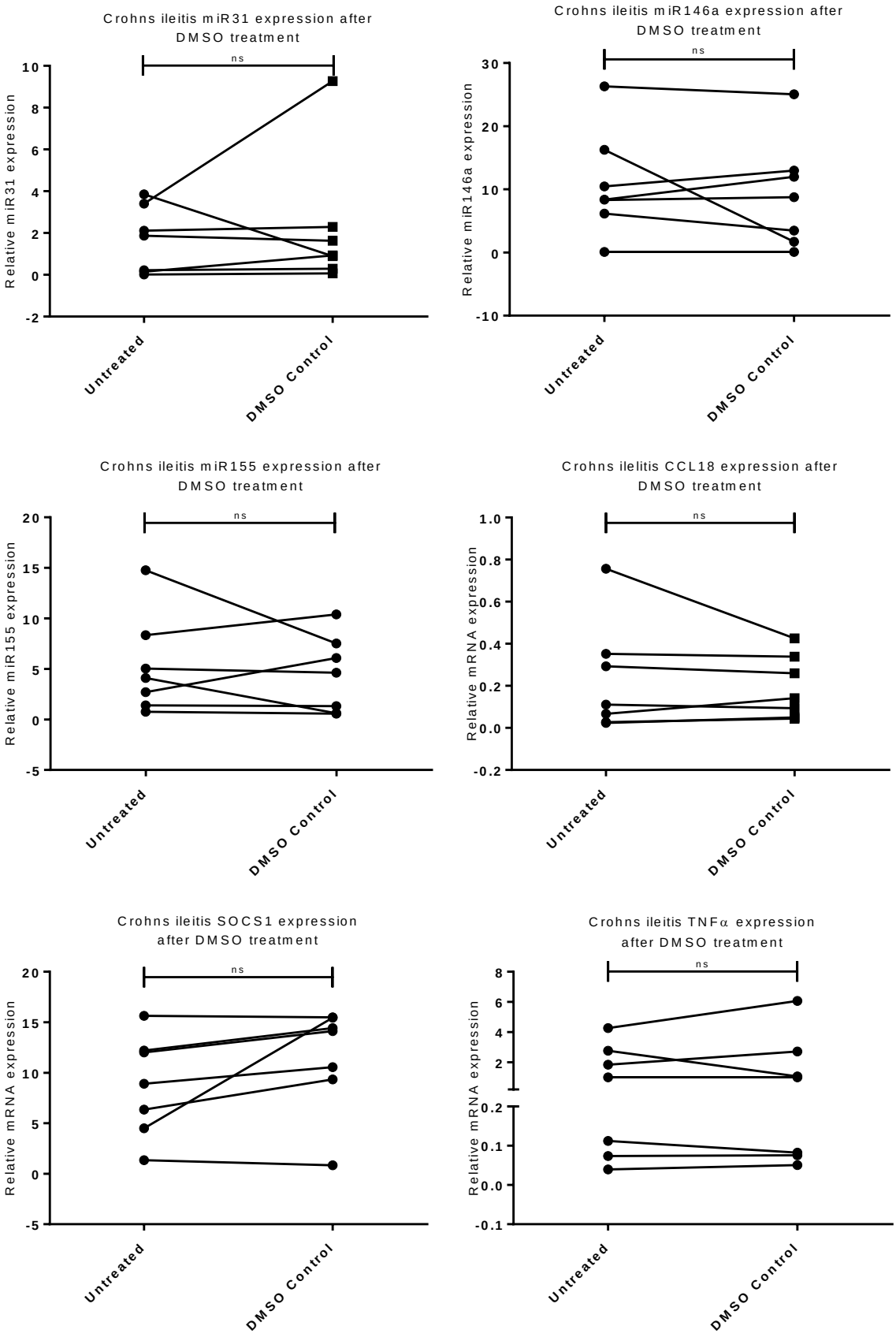


Figure 48. Ex vivo model showing the effects of DMSO on expression in active Crohn's Ileitis. n=7

3.2.6 Normal Colonic Mucosa

3.2.6.1 microRNA profiles in normal mucosa are not altered by drug treatments in an ex vivo model

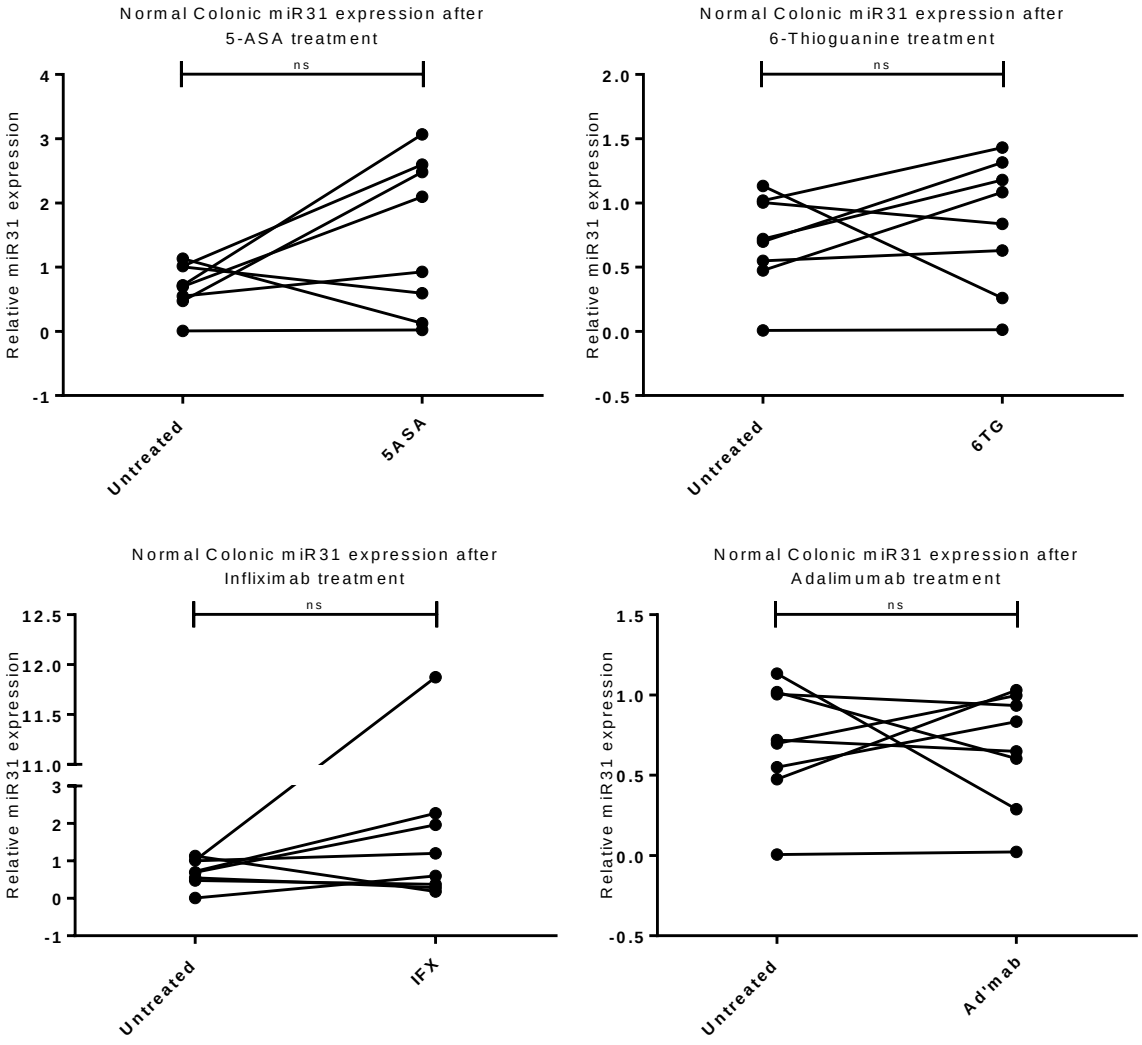


Figure 49. Ex vivo model showing the effects of treatment on miR-31 expression in normal colonic mucosa. n=8

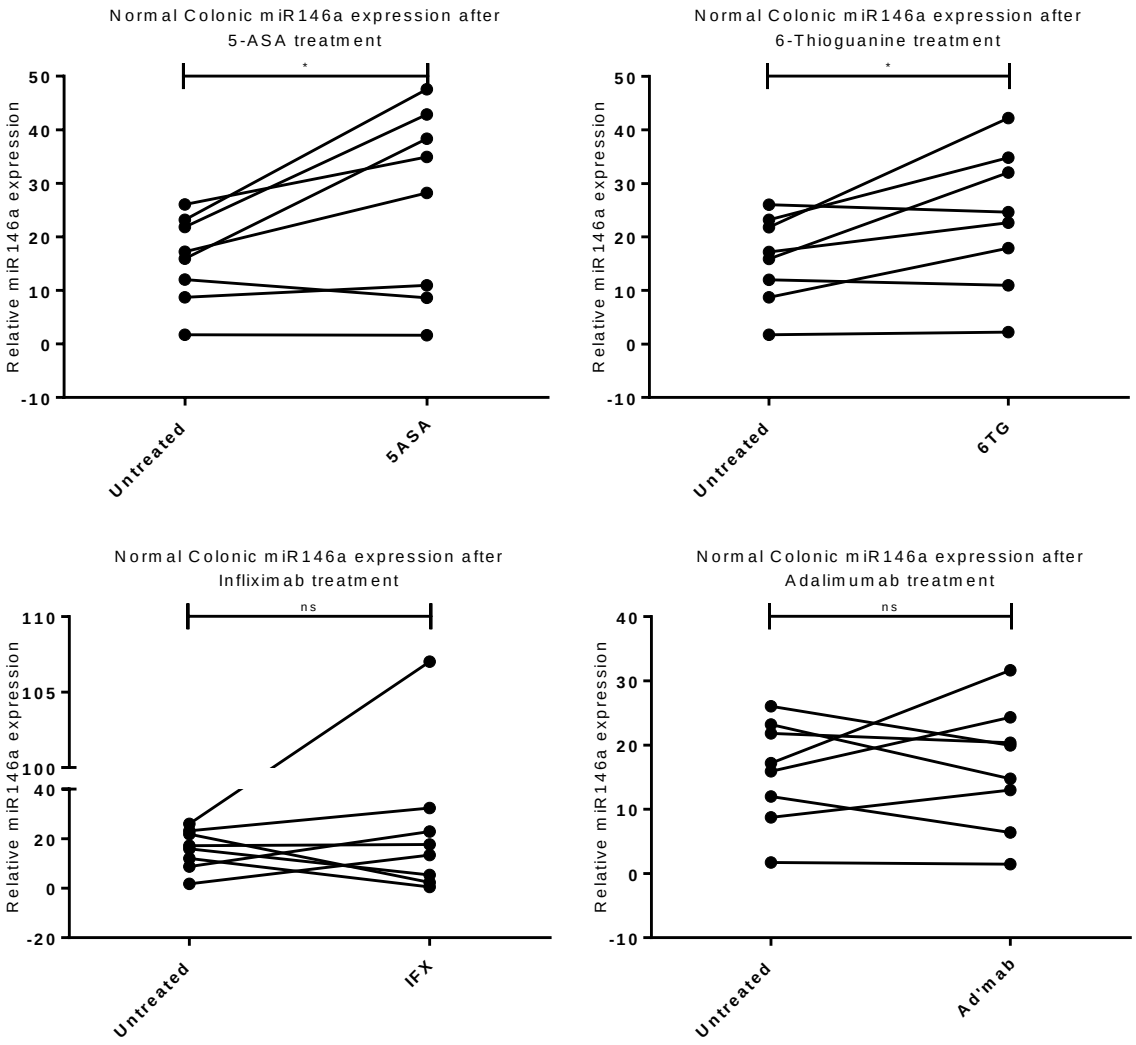


Figure 50. Ex vivo model showing the effects of treatment on miR-146a expression in normal colonic mucosa. n=8

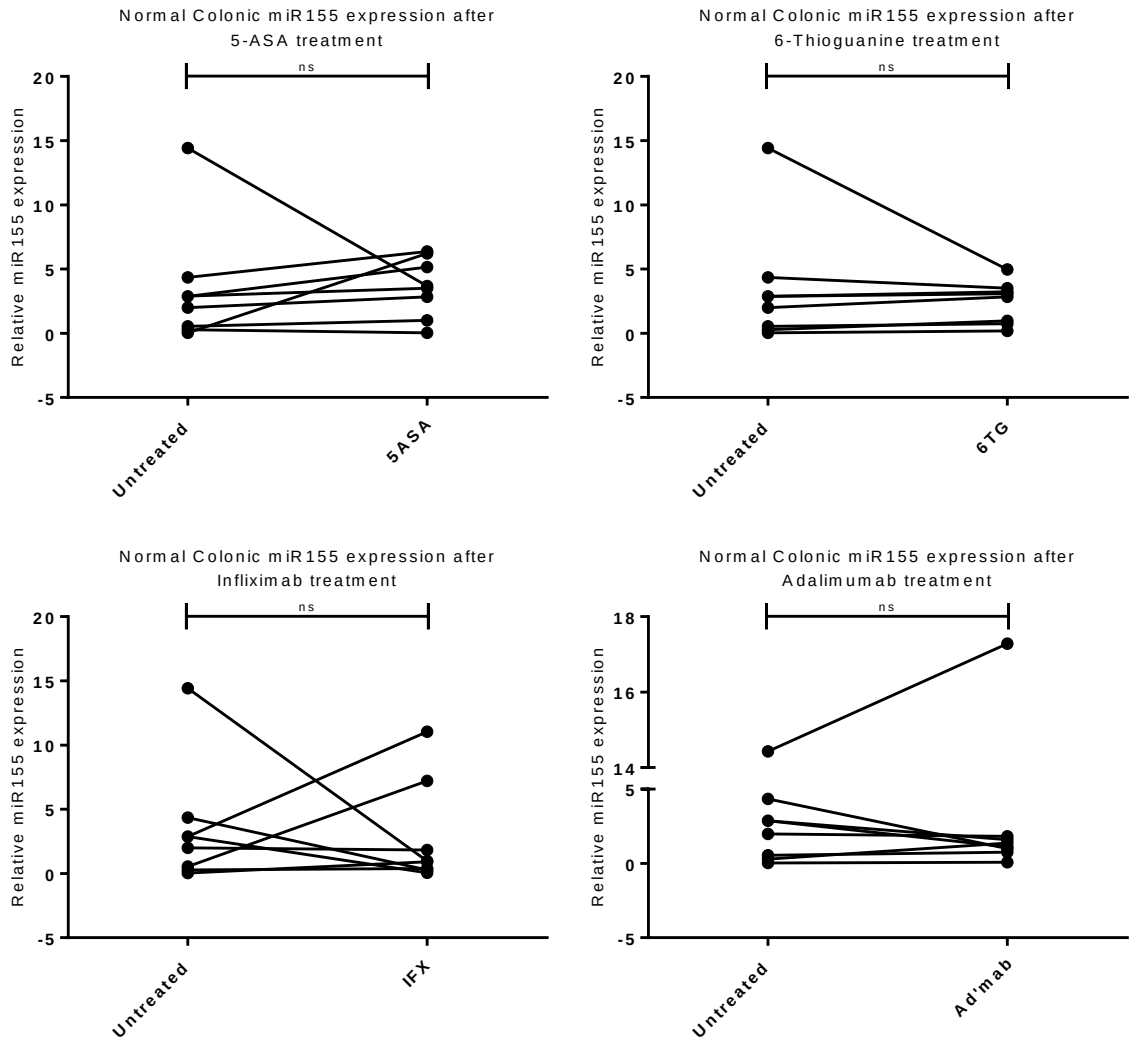


Figure 51. Ex vivo model showing the effects of treatment on miR-155 expression in normal colonic mucosa. n=8

The treatments used do not affect miR-31 expression in normal uninflamed colonic mucosa.

In Normal uninflamed colonic mucosa, treatment with 5-ASA and 6-Thioguanine significantly increase the expression of miR-146a in the mucosa. This effect is not seen with the anti-TNF treatments.

The treatments used do not affect miR1-55 expression in normal uninflamed colonic mucosa.

3.2.6.2 Cytokine profiles in normal mucosa are not altered by drug treatments in an ex vivo model

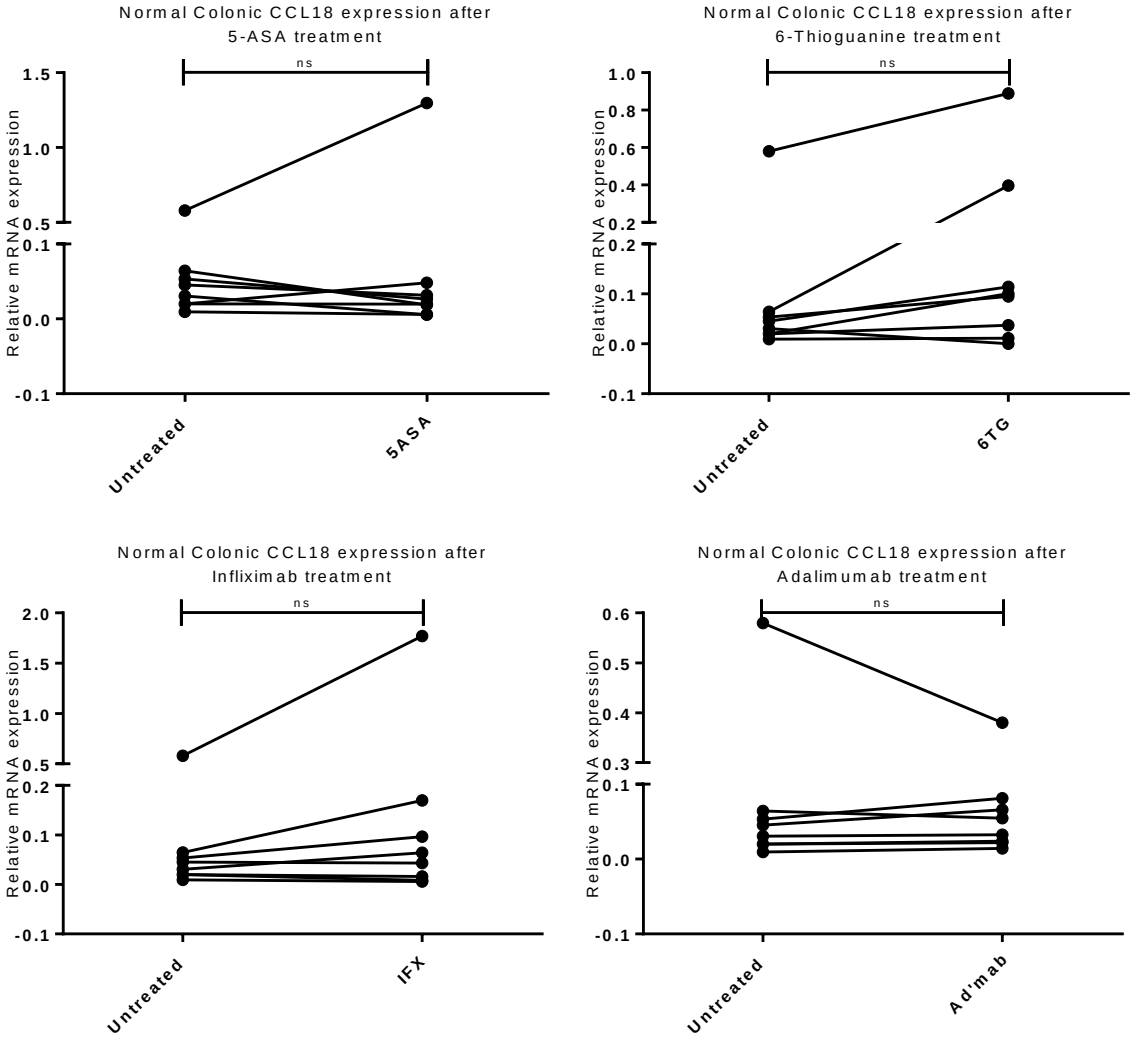


Figure 52. Ex vivo model showing the effects of treatment on CCL18 expression in normal colonic mucosa. n=8

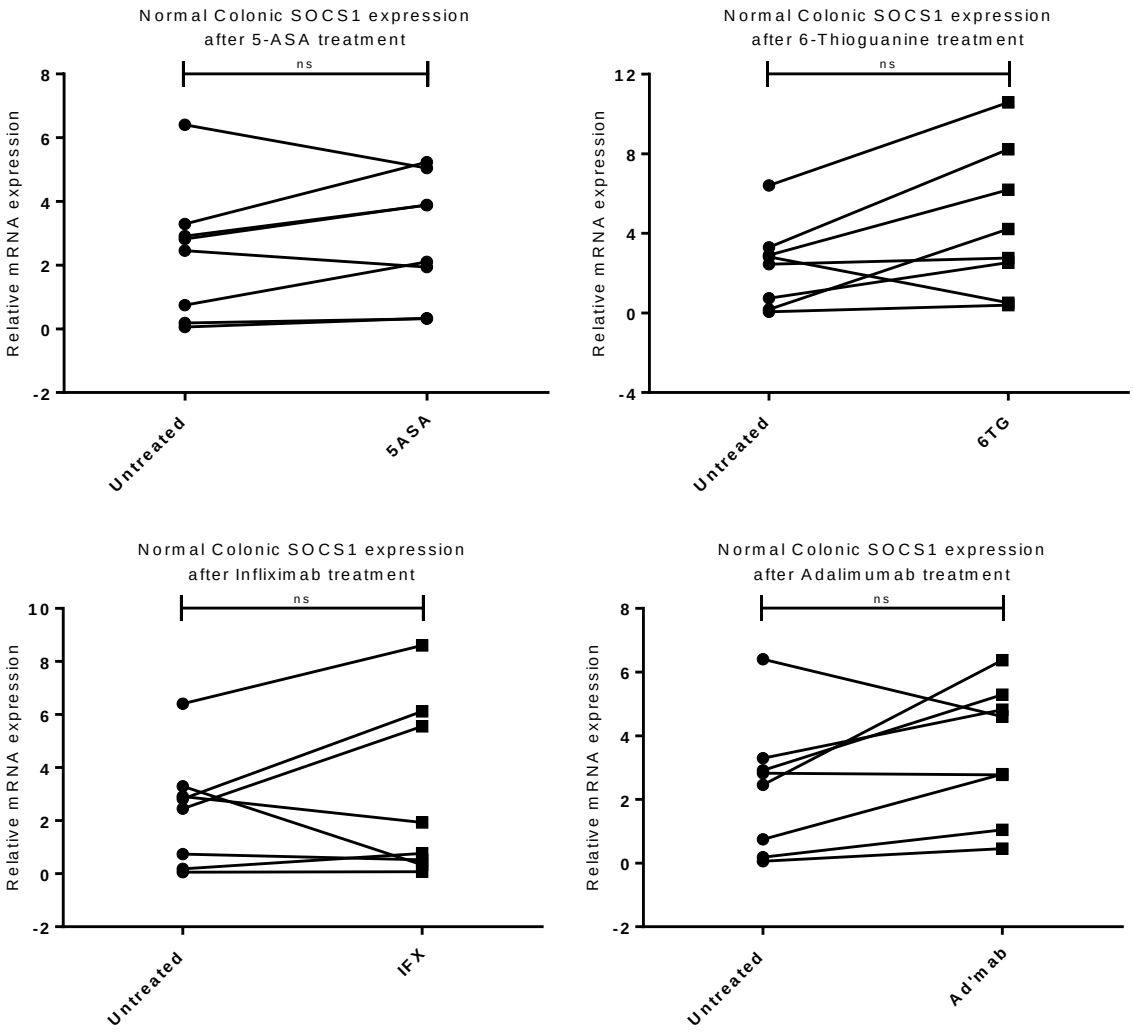


Figure 53. Ex vivo model showing the effects of treatment on SOCS1 expression in normal colonic mucosa. n=8

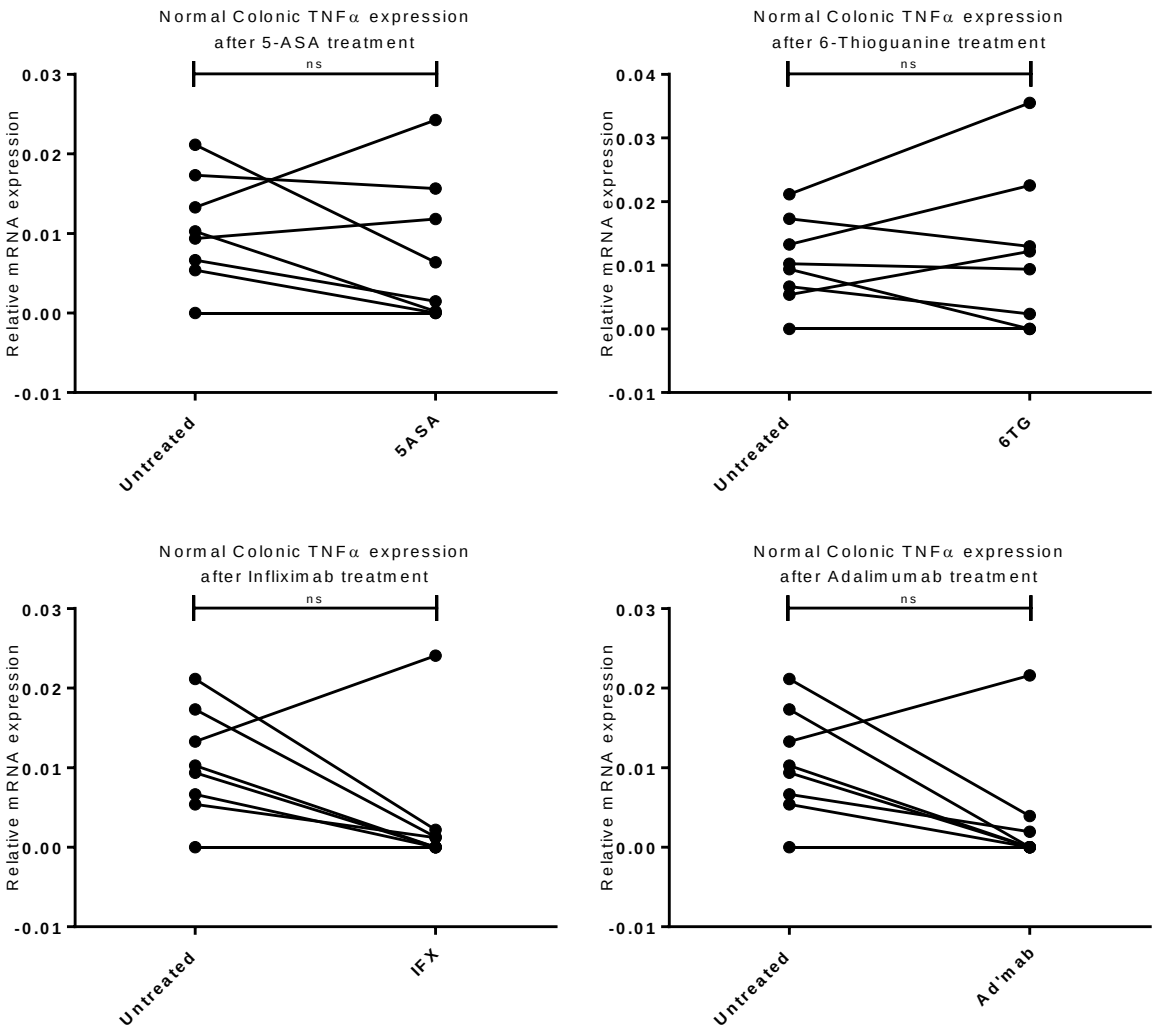


Figure 54. Ex vivo model showing the effects of treatment on TNFα expression in normal colonic mucosa. n=8

The treatments used do not affect CCL18, SOCS1, or the already very low TNFα expression in normal uninflamed colonic mucosa.

3.2.6.3 DMSO Control Treatments

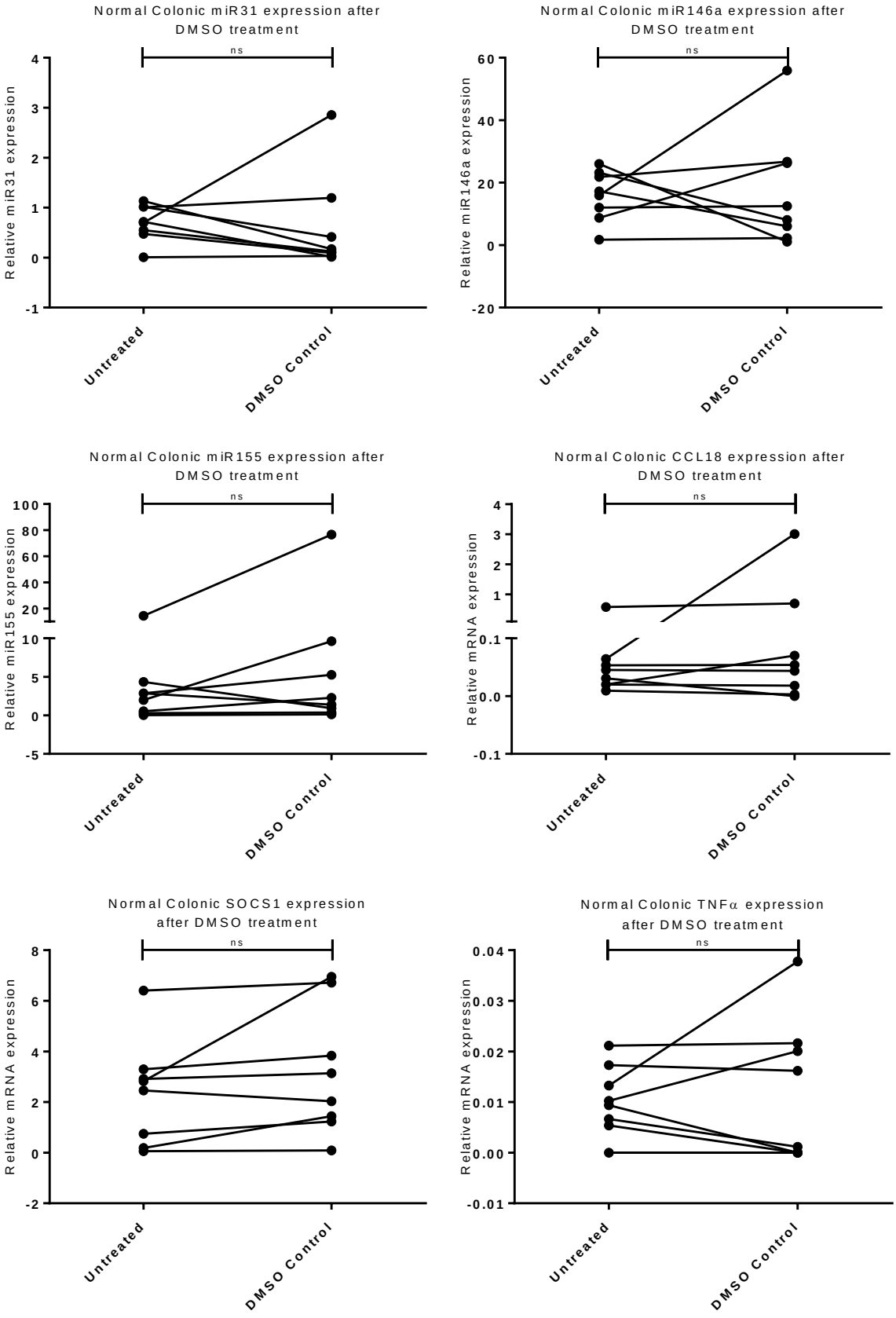


Figure 55. Ex vivo model showing the effects of DMSO on expression in normal colonic mucosa. n=8

3.2.7 Discussion

These experiments have shown for the first time how specific treatment for IBD influence the expression of specific microRNAs and cytokines in biopsies taken from a well-defined, homogeneous group of patients.

6-Thioguanine, the active metabolite of Azathioprine, lowers microRNAs 31, 146a and 155 in the colonic mucosa of active UC and Crohn's disease. It also lowers TNFα and CCL18 in the colonic mucosa of active UC and Crohn's disease. However, in the ileal mucosa of active Crohn's ileitis, 6-TG only reduces the expression of miR-155 and TNFα. The expression of these microRNAs and cytokines are not altered in normal colonic mucosa when it is exposed to 6-TG. SOCS-1 expression is not altered in colonic mucosa by any of the treatments used in either UC, Crohn's colitis or normal mucosa, however SOCS-1 expression is reduced in the active Crohn's ileitis, after the treatment of all the drugs used.

Anti-TNF biological therapies lower miR-155 and TNFα expression of active Crohn's colitis, but this is less obvious in UC. Infliximab does appear to lower miR-31 expression in active Crohn's colitis but not in active UC. The pattern is the same with Adalimumab, however significance has not been reached in Crohn's colitis. CCL18 expression is also reduced by anti-TNF therapies in both UC and Crohn's colitis except with Adalimumab in active UC, which cannot be explained. In normal colonic mucosa anti-TNF therapy does not replicate these effects.

In Crohn's Ileitis, miR-155 expression is again reduced by anti-TNF therapy. This pattern is also seen with TNFα expression in Infliximab treated ileal biopsies, however with Adalimumab treatment TNFα expression reduction has not achieved significance although there is a trend towards significance. miR-31 expression is not reduced by anti-TNF therapy in active Crohn's ileitis, in fact miR-31 expression is significantly increased in the group of biopsies treated with Adalimumab. CCL18 has not been altered in the actively diseased ileal mucosa, unlike that seen in colonic mucosa. In this experiment SOCS1 expression in actively diseased ileal mucosa is significantly decreased by anti-TNF therapy, and by 6-TG and 5-ASA as already mentioned. This was not seen in colonic mucosa.

These results confirm that the specific treatments used for IBD alter the microRNA and cytokine profiles in different ways. This fact is important to note when previous studies have

analysed microRNA expression in heterogeneous groups on various treatments. The treatments also change expression in different ways depending on the classification of IBD being treated. This confirms the different molecular pathogenesis between Crohn's and UC, and between Colonic and small bowel Crohn's disease. This model could be used in the future to predict disease response to therapy and aid personalised treatment strategies.

This model however could be improved. The concentrations of drugs used in the model was based on concentrations measured in target tissue in other studies, and also estimated pragmatically. I did not measure the tissue drug concentration but estimated the drug concentrations I was going to use. Isotope labelling and immunofluorescence of the drug in the target tissue is something to consider if this were to be repeated.

The time of the incubation period was chosen for pragmatic reasons. Firstly, to avoid bacterial and fungal contamination of the tissue culture, and also as no known time period of incubation was known to be effective an arbitrary time point was chosen.

Future models could examine whether different incubation times and drug doses could influence the results obtained.

3.3 MicroRNA and cytokine expression in Ulcerative Colitis before and after treatment with Tralokinumab

3.3.1 Introduction

Ulcerative colitis shows a particular immune response favouring an atypical Th2 pathway, and the increased secretion of IL-13(*211*). IL-13 could be the cytokine that is responsible for the dysregulation of the epithelial barrier and tight junctions that leads to the increased permeability that could introduce antigens to the lamina propia causing inflammation(*23*). This pathway is dependent on many other genes such as chemokine ligands 18 and 26 (CCL18) (CCL26) and suppressor of cytokine signalling 1 (SOCS1).

Tralokinumab is a humanised monoclonal antibody targeting the IL-13 cytokine. A phase II, multicentre, double blind, placebo controlled, randomised controlled trial has been conducted to look at the safety and efficacy of this drug in moderate to severe Ulcerative Colitis(*202*).

The primary outcome of this experiment was to ascertain if a panel of miRNAs (miR-31, 146a and 155) which we have been shown to target IL13Rα1 have utility as biomarkers to predict response, mucosal healing, and remission in patients with moderate to severe Ulcerative Colitis treated with Tralokinumab. Secondary outcomes were to gain further insights into the mechanism of action of Tralokinumab as well as the role the IL-13 pathway may play in the pathogenesis of UC.

RNA extracted from colonic mucosal biopsies from 72 patients recruited into the phase II study Evaluation of Efficacy and Safety of Tralokinumab in Patients with Active, Moderate to severe Ulcerative Colitis(*202*), were used for gene and microRNA analysis**.**

The original study design used non-hospitalised adults with UC (total Mayo score ≥6). These patients were randomised to receive tralokinumab 300 mg or placebo subcutaneously every 2 weeks for 12 weeks. Clinical response at week 8 was measured as the primary end-point. More robust secondary end points of clinical remission and mucosal healing rates at week 8 were also recorded.

In patients with distal UC, biopsies were taken from both affected (inflamed) tissue and areas more proximal which were unaffected (non-inflamed). Thirty-four patients received placebo whilst 38 received fortnightly 300mg subcutaneous Tralokinumab. Colonic mucosal

biopsies were taken from the sigmoid colon at baseline before treatment was commenced and at week 12. The patient demographics are seen in table 25. The data demographic data collected in this study was limited compared to the data collected from patients in the previous section, however the age and average Mayo score in this study is comparable to that of the UC patient cohorts in section 3.1 and 3.2.

RNA samples were extracted using RNeasy minikit. Reverse transcription and Real Time qPCR were performed to analyse the relative expression of genes and microRNAs.

Characteristics	Placebo n=34	Tralokinumab n=38
Mean Age, Years (SD)	41 (14)	44 (11)
Mean BMI, Kg/M^2 (SD)	24.6 (4.2)	24.4 (4.2)
Male, N (%)	15 (47)	16 (42)
Caucasian, N (%)	34 (100)	37 (97)
Mean Total Mayo Score (SD)	8.3 (1.3)	8.5 (1.6)

Table 26. Baseline demographics from 72 randomised patients. Data provided by AstraZeneca

3.3.2 MicroRNA expression

The relative fold expression of each microRNA analysed has been shown in the Figures below, and then the important microRNA discussed in more detail.

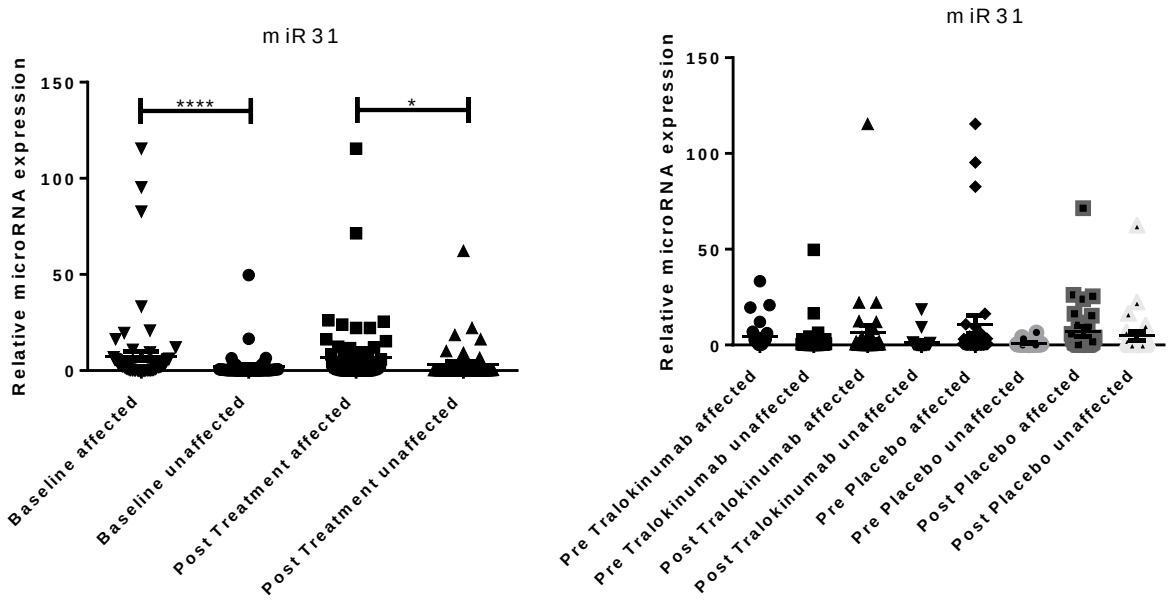


Figure 56. miR-31 levels in affected and unaffected tissue and the breakdown of the placebo vs active drug groups

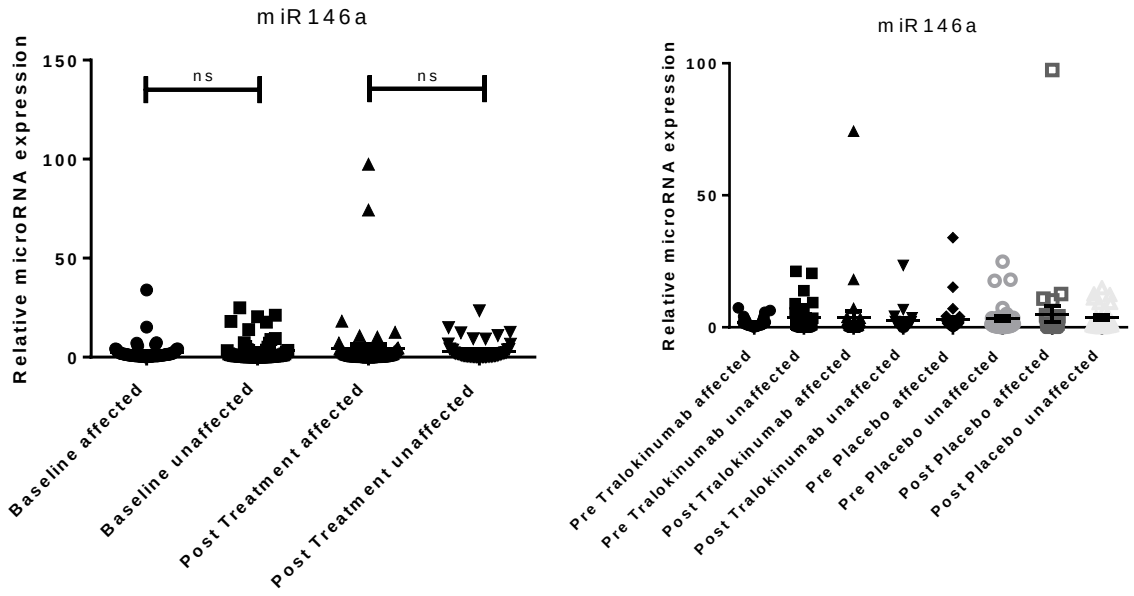


Figure 57. miR-146a levels in affected and unaffected tissue and the breakdown of the placebo vs active drug groups

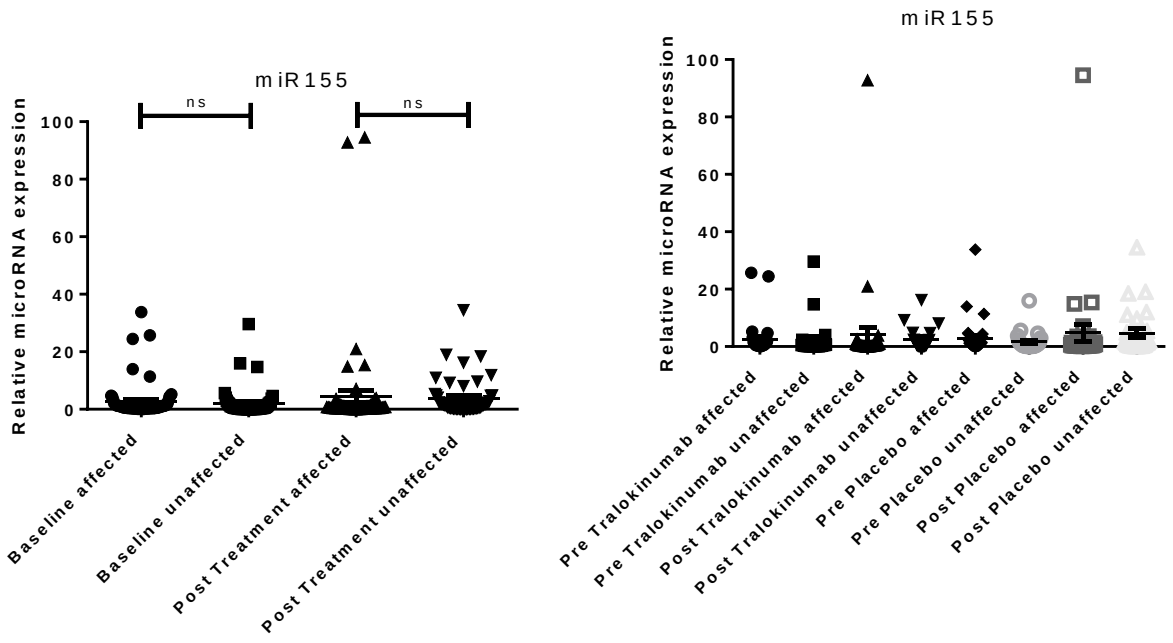


Figure 58. miR-155 levels in affected and unaffected tissue and the breakdown of the placebo vs active drug groups

Only miR-31 showed significant differences between the affected and unaffected tissues so this was analysed further to see if the pre-treatment levels of miR-31 could act as a biomarker to predict treatment response, clinical remission, and mucosal healing. (Figure 59. 60. 61.)

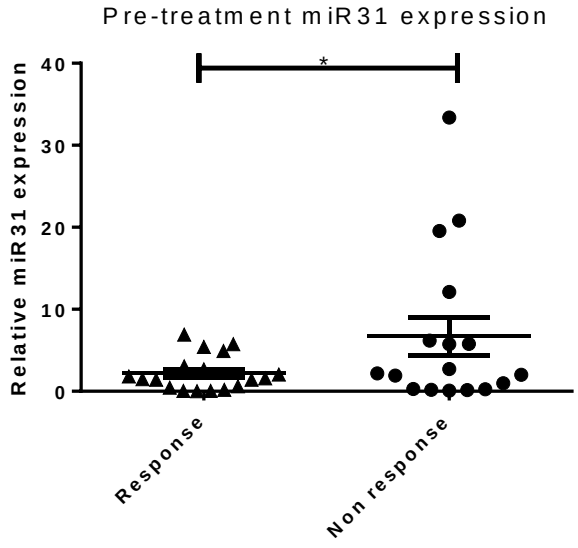


Figure 59. The levels of miR-31 in inflamed tissue can predict the Non-response to Tralokinumab

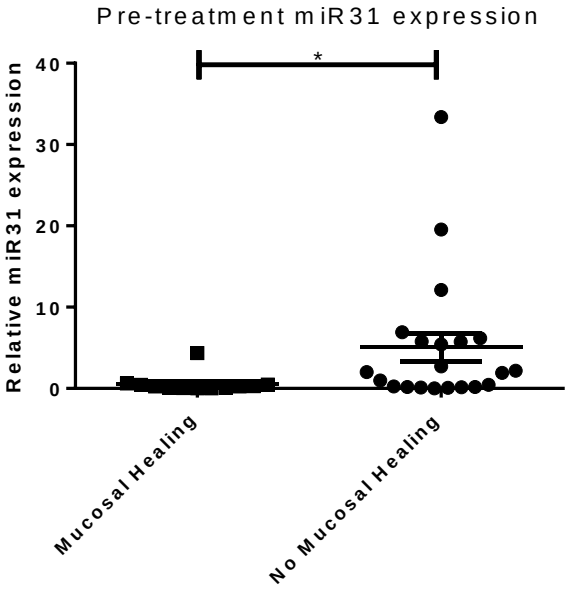


Figure 60. The levels of miR-31 in inflamed tissue can predict patients who will not develop Mucosal Healing with Tralokinumab

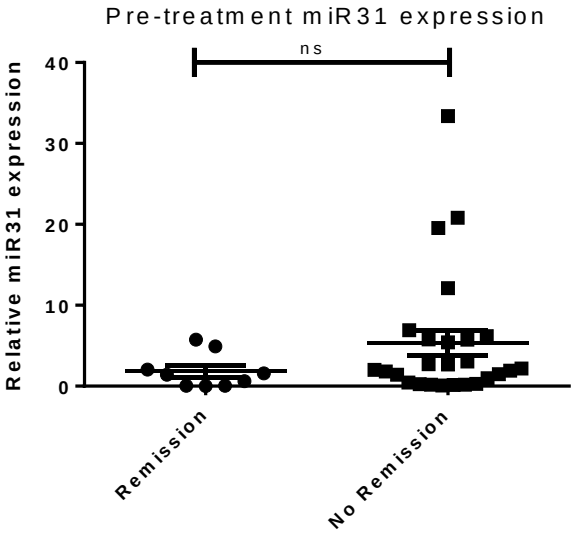


Figure 61. The levels of miR-31 in inflamed tissue can possibly predict patients who will not develop Remission with Tralokinumab

When this analysis was performed for miR-146a and miR-155 the levels did not show biomarker predictions.

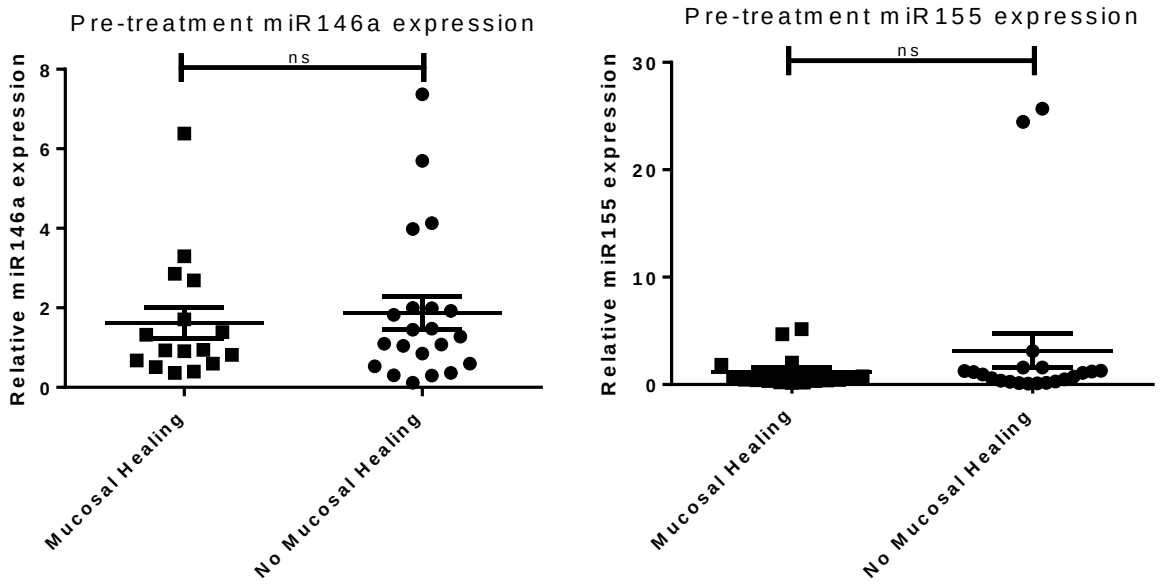


Figure 62. The levels of miR-146a and miR-155 in inflamed tissue cannot predict patients who will or will not develop Mucosal Healing with Tralokinumab

The effect of a reduction in miR-31 in actively inflamed mucosa that undergoes mucosal healing is confined to the patients who are treated with Tralokinumab, rather than those who develop spontaneous (placebo group) mucosal healing (Figure 63).

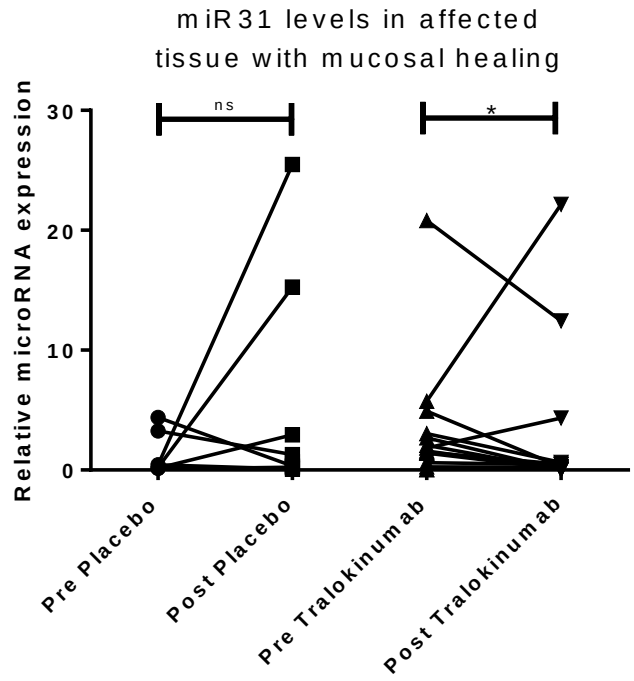


Figure 63. The levels of miR-31 expression are reduced when mucosal healing is achieved but only in those patients who have achieved this with Tralokinumab therapy

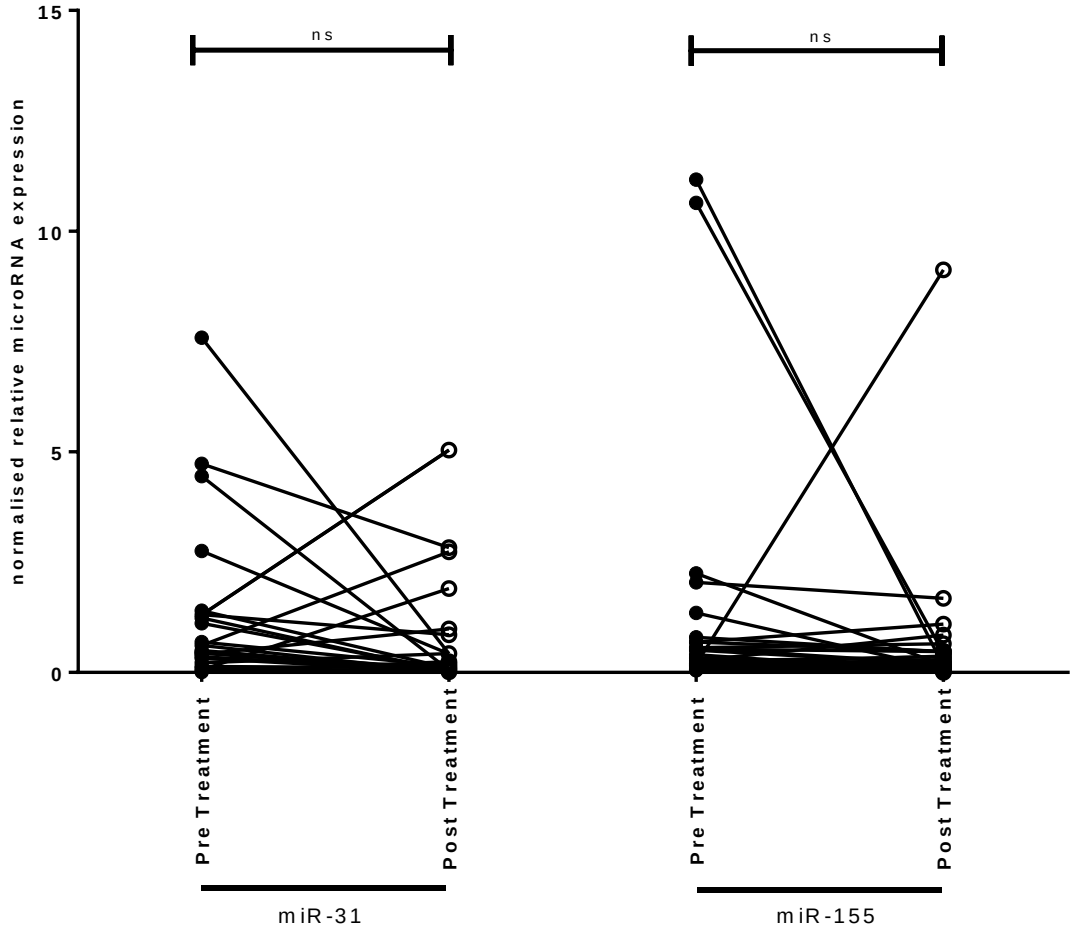


Figure 64. Summary graph of the effects of microRNA-31 and microRNA-155 expression in colonic mucosa of all patients receiving Tralokinumab regardless of response.

3.3.3 Gene expression

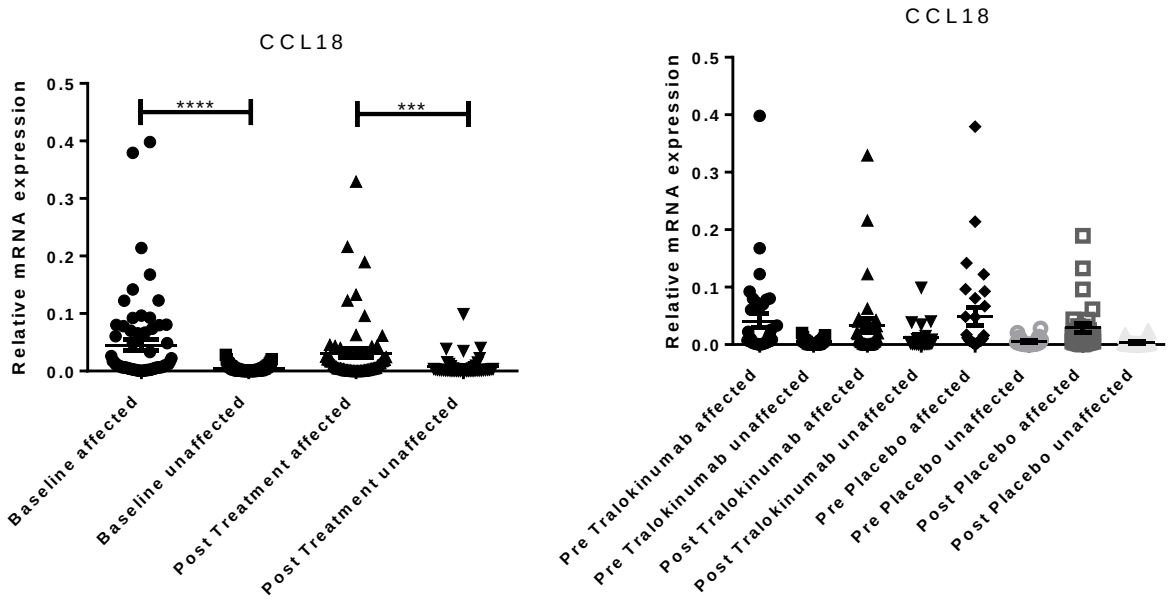


Figure 65. CCL18 mRNA levels in affected and unaffected tissue and the breakdown of the placebo vs active drug groups

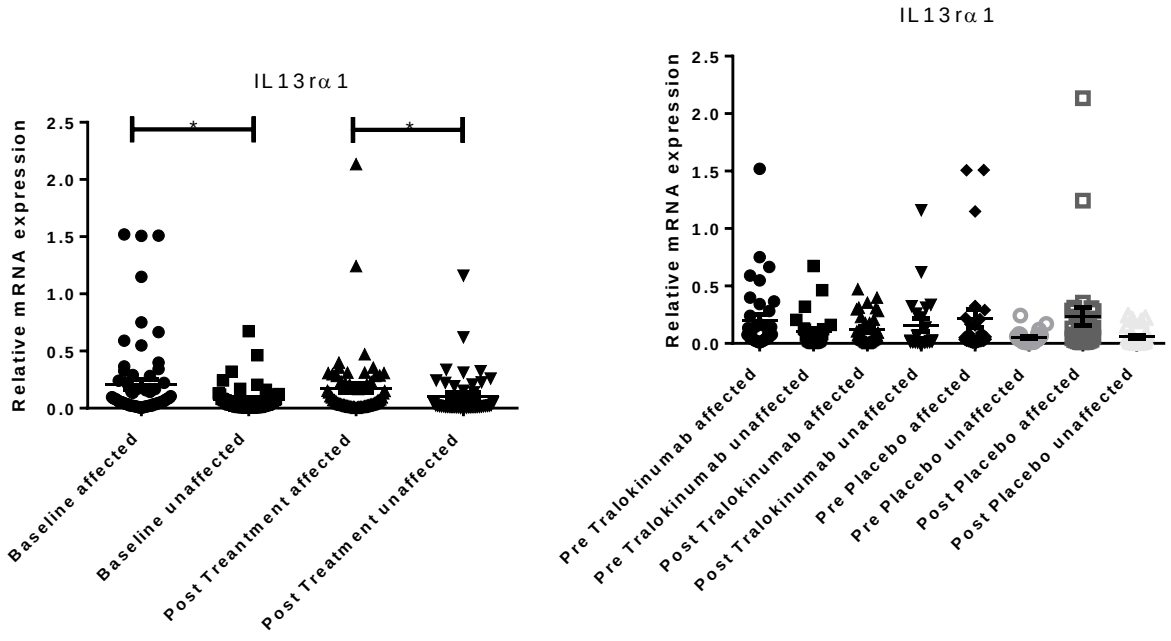


Figure 66. IL13rα1 mRNA levels in affected and unaffected tissue and the breakdown of the placebo vs active drug groups

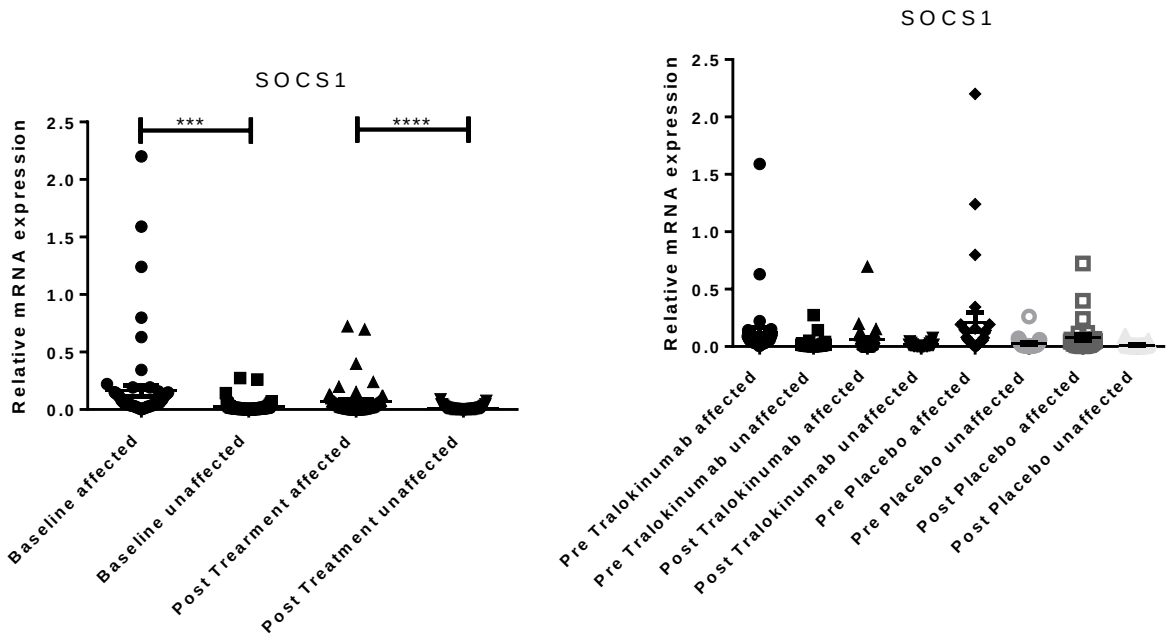


Figure 67. SOCS1 mRNA levels in affected and unaffected tissue and the breakdown of the placebo vs active drug groups

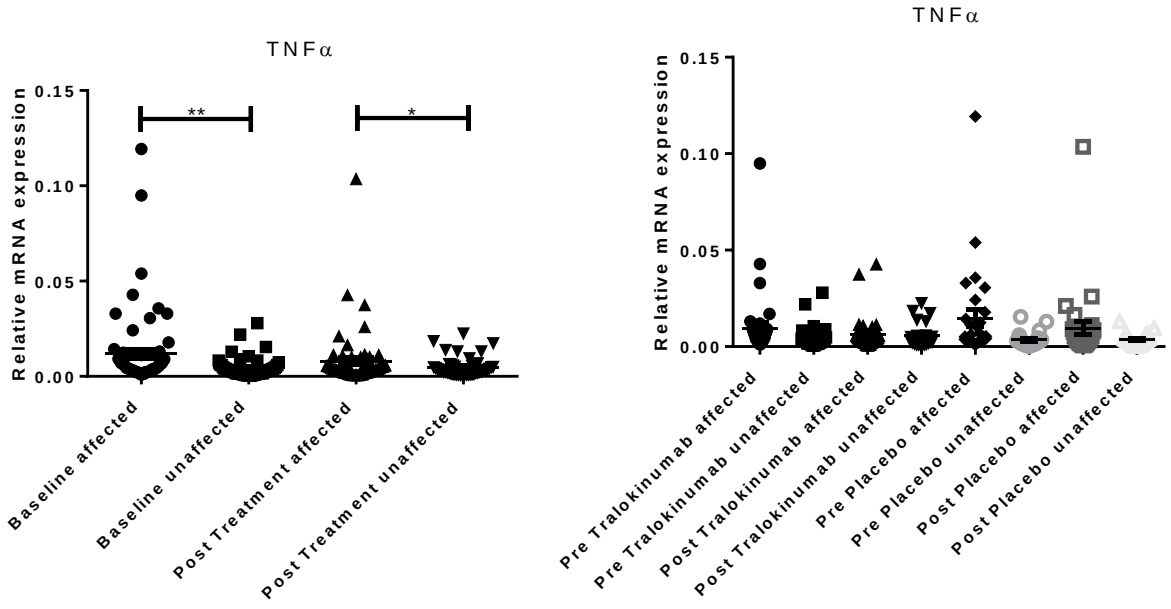


Figure 68. TNFα mRNA levels in affected and unaffected tissue and the breakdown of the placebo vs active drug groups

Pre-treatment levels of mRNA for all these genes could not predict outcome except when using IL13rα1 mRNA levels to predict clinical remission (Figure 69).

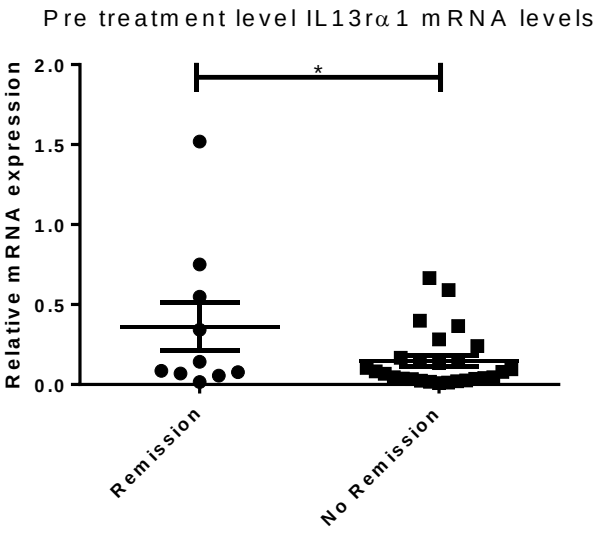


Figure 69. The levels of IL13rα1 mRNA in inflamed tissue can possibly predict patients who will develop remission with Tralokinumab

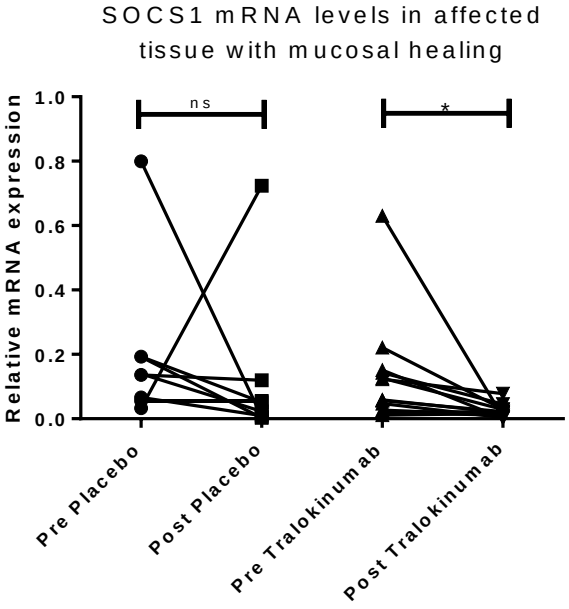


Figure 70. The levels of SOCS1 mRNA expression are reduced when mucosal healing is achieved but only in those patients who have achieved this with tralokinumab therapy

All the genes analysed have significant up regulation in inflamed tissue when compared to unaffected tissue. SOCS1 mRNA levels are significantly down regulated when mucosal healing is achieved but only in those patients who were treated with Tralokinumab. There was no significant difference in SOCS1 levels in patients who achieved spontaneous mucosal healing (Figure 70).

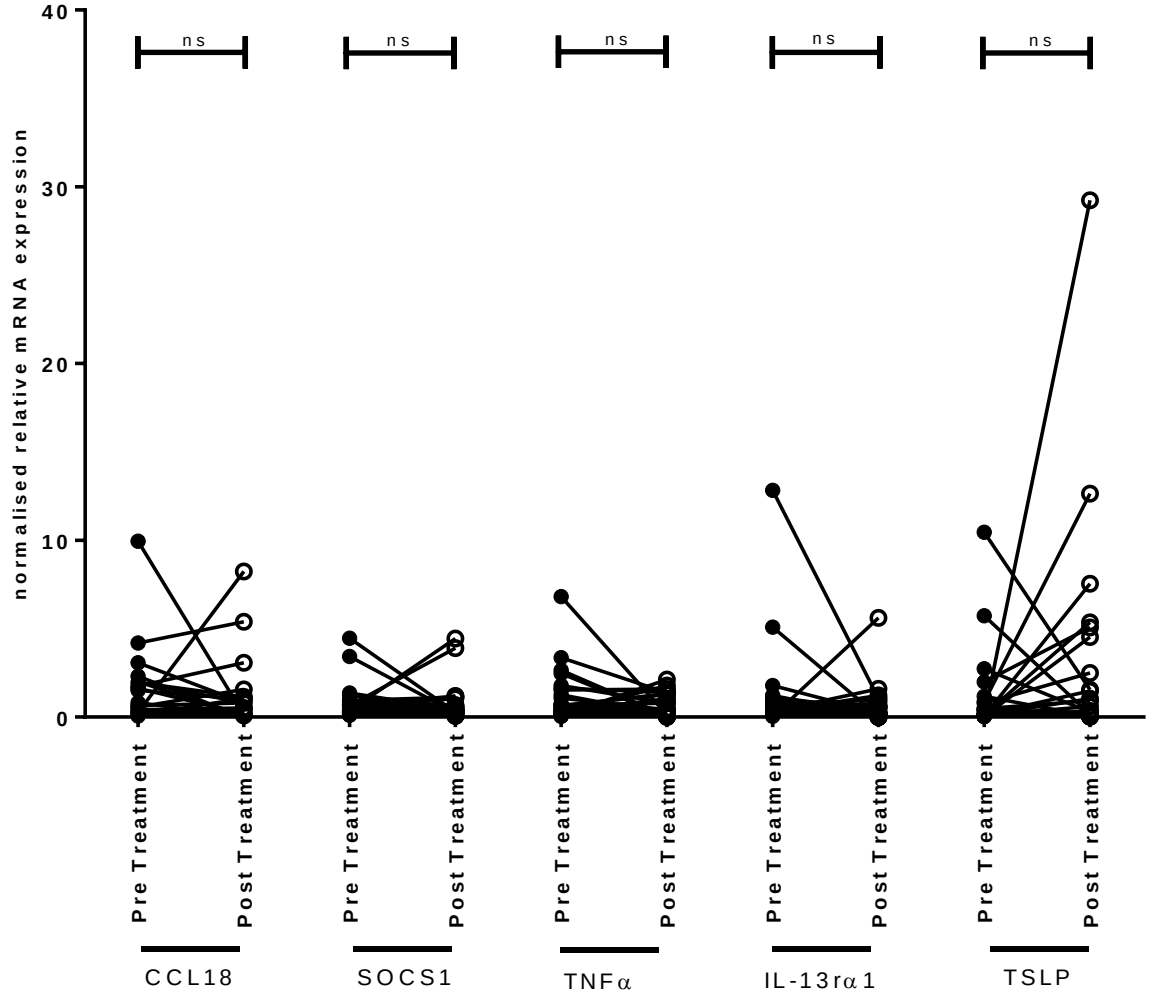


Figure 71, Summary of the variance of gene expression in colonic mucosa of all patients receiving Tralokinumab, regardless of response.

3.3.4 Discussion

miR-31 seems to be the most important microRNA of the panel analysed in active UC and perhaps may be important in the mechanism of action of Tralokinumab in moderate to severe UC. In our analysis, miR-31 was the only miRNA or gene which showed any potential as a biomarker to predict whether clinical response or mucosal healing could be achieved in response to Tralokinumab therapy. If patients with raised mir-31 were excluded from the analysis, clinical response rates increased from 47% to 52%, mucosal healing rates from 44% to 57%, and clinical remission from 26% to 32%. When applying the same corrections on the placebo arm of the study, there was no significant increase in rates of these responses. We analysed whether combining the miR-31 with other markers (CCL18, IL13Ra1 etc.) would improve these rates further in the Tralokinumab group, but every combination tested was not able to increase response rates beyond that of miR-31 alone.

miR-31 expression was significantly down regulated in those patients who achieved mucosal healing with Tralokinumab but not patients who achieved this in the placebo group. Thus, this down regulation was independent of mucosal healing as it was not seen in those patients with spontaneous mucosal healing (placebo). The importance of miR-31 downregulation after Tralokinumab treatment may be underestimated as some of those patients treated with Tralokinumab may have achieved spontaneous healing despite the treatment (and thus have shown improvement without miR-31 reduction). The length of remission achieved in placebo patients who did not reduce miR-31 levels would have been interesting to follow up for longer than the study design permitted, as the miR-31 levels maybe be time dependant and, in some patients, have not been reduced to the full potential.

This particular result offers insights into the mechanisms of action of Tralokinumab in UC which seems to directly affect the IL-13 inflammation of the gut, as reflected by the levels of miR-31, a microRNA linked with IL-13 inflammation. In agreement with this, we also observed that cytokine suppressor gene SOCS1, was downregulated exclusively in the Tralokinumab group of patients that benefited from mucosal healing. SOCS1 is a classic IL-13 dependent gene, and a marker of “Th2-type inflammation”.

The reduction of SOCS1, together with miR-31, suggest that Tralokinumab, as expected, has a therapeutic effect through the suppression of the IL-13 inflammation in UC patients. This effect can be followed specifically in the Tralokinumab group that achieve mucosal healing (miR-31 and SOCS1 expression), as opposed to other more generic markers (such as CCL18 and IL13Ra1), that are also present in the placebo group that showed mucosal healing.

miR-31 may give an insight into why Tralokinumab did not have a higher response rates in a study designed to assess its efficacy as an induction agent as opposed to a maintenance agent. Our data shows that patients with high expression of miR-31 responded poorly to Tralokinumab. We have demonstrated clear evidence of IL-13 mediated inflammation in active ulcerative colitis, but not the presence of the therapeutic target of Tralokinumab in the gut mucosa at the point in time that the drug was administered. IL-13 expression may be upregulated early in the disease process by an unknown trigger, initiating mucosal damage and activating Th2 inflammatory pathways (CCL18, SOCS1 etc.) Increased miR-31 expression may reflect the later stages of an IL-13 induced inflammatory process, where IL-13 has been down regulated by miR-31 as part of a negative feedback loop, thus removing the therapeutic target for Tralokinumab. miR-31 targets IL13Rα1 and this reduces sensitivity to IL-13, but bioinformatics suggests that miR-31 may also reduce IL-13 expression as well. This hypothesis is strengthened by the difficulties in actually measurement of IL-13 either by ELISA or mRNA expression by RTqPCR in inflamed tissue(*208*). Therefore, patients would not benefit from an anti-IL-13 drug as an induction agent as the therapeutic target has been down-regulated soon after the initial trigger event for the flare, perhaps even before the patient becomes symptomatic. On this basis it is possible that anti-IL13 drugs may be potent maintenance agents rather than induction agents. The design of the current trial does not allow this hypothesis to be further explored.

3.4 The role of Thymic Stromal Lymphopoeitin (TSLP)

3.4.1 Introduction

Bioinformatic searches and our own qPCR correlations in active UC tissue have shown that miR-31 and miR-155 target import genes within the IL-13 dependant pathway.

TSLP is predicted to be one such target of miR-31 that plays an important role in the IL-13 dependant pathway(*212*). TSLP is a IL-7 like cytokine that is critical in the establishment of Th2 type inflammation(*213, 214*), it is induced by IL-13 and attracts T-cells by inducing T-cell attracting cytokines from monocytes. It has been shown that TSLP deficient mice fail to develop allergic inflammation in response to inhaled antigen(*215, 216*).

I use the samples from the clinical trial(*202*) to investigate the expression of TSLP before and after treatment with Tralokinumab to show if TSLP can be used to predict disease response, and whether there is differential expression in inflamed and uninflamed tissue, and show a relationship with it and its predicted target, miR-31. The patient demographics are shown in table 25 from the previous chapter.

I then go on to analyse the difference in TSLP protein expression in sigmoid mucosa in active UC compared to inactive and healthy mucosa by ELISA, and normalise for biopsy volume using a BCA protein assay.

3.4.2 TSLP mRNA expression in the mucosa of active UC is decreased compared to inactive disease and healthy mucosa

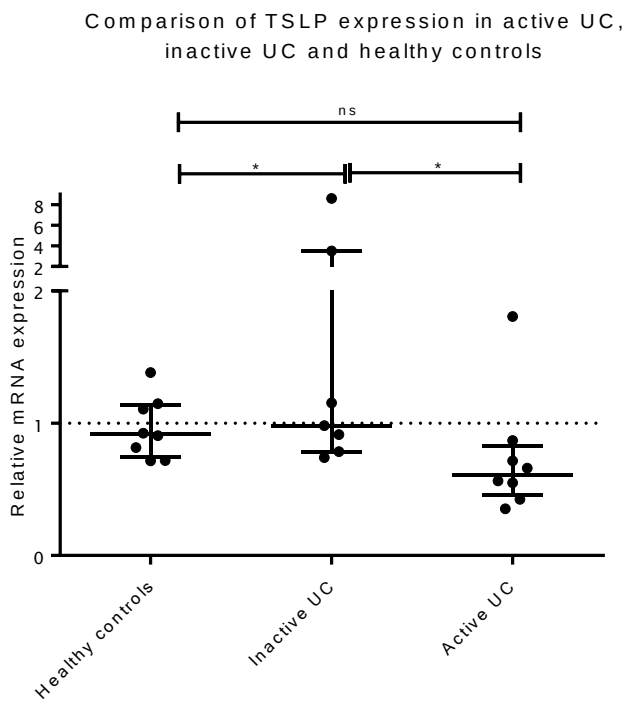


Figure 72. The relative expression of TSLP mRNA in the sigmoid mucosa. The expression of TSLP mRNA in active and inactive sigmoid UC was compared against healthy controls. This data was taken from Dr Andrew Claridge.

The expression of TSLP mRNA is significantly decreased by 0.6118-fold (IQR 0.4558 – 0.8301), in the mucosal biopsies of active sigmoid UC compared with inactive UC and healthy controls.

There is no difference in the expression of TSLP in the sigmoid mucosa of inactive UC compared with healthy controls.

3.4.3 The effects of conventional therapy on TSLP expression in active UC

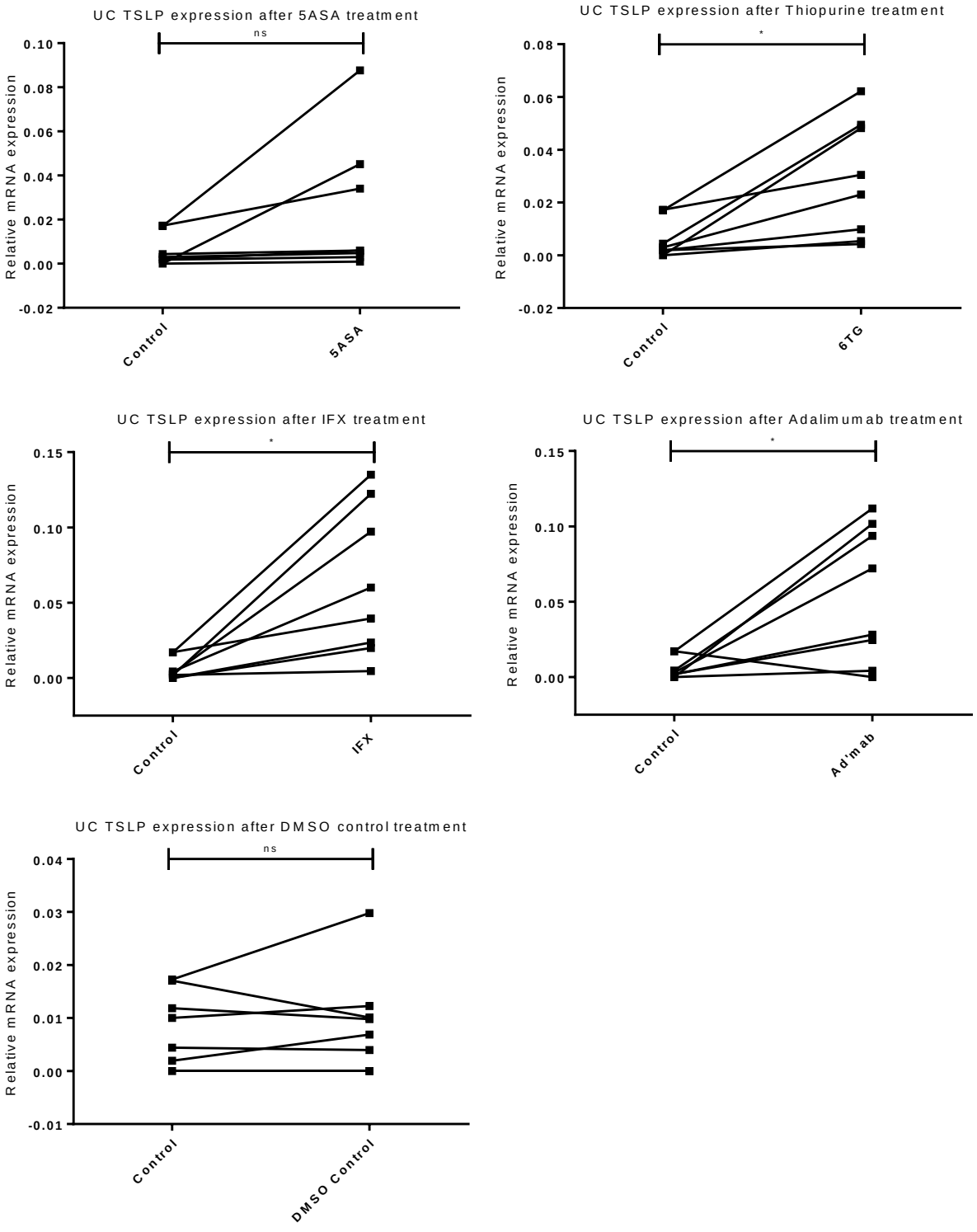


Figure 73. Ex vivo model showing the effects of conventional therapy on TSLP expression in moderate to severe active UC. The patient demographics are show in table 25, in chapter 3.2.2

Figure 73 shows that ex vivo treatment of active UC mucosal tissue with the Thiopurine metabolite 6-Thioguanine, and biological therapies Infliximab and Adalimumab, upregulates TSLP expression. As seen in Figure 70, TSLP is upregulated in inactive UC and healthy mucosa when compared to TSLP

expression in the mucosal biopsies of active UC. Therefore, the treatments with 6-Thioguanine, Infliximab and Adalimumab are influencing the TSLP expression in active UC towards that seen in inactive disease and healthy controls.

The demographic of the patients used in this experiment are show in table 25, in chapter 3.2.2.

3.4.4 TSLP protein expression in the sigmoid mucosa by ELISA

Samples are matched for age, sex and ethnicity. All patients in the active UC group had Baron grade 3 macroscopic inflammation and are treatment naive. CRP was significantly higher in the active UC group compared with healthy control. The patient characteristics are shown in table 27.

Characteristics	Control (n=12)	Inactive UC (n=12)	Active UC (n=11)
Age	58 (43 – 70)	55 (48 – 67)	49 (29-63)
Sex (F/M)	6/6	6/6	5/6
Ethnicity (if not white)	0	0	0
Smoker / Ex-Smoker	3x ex-smoker 9 x non-smoker	2x ex-smokers 1x smoker 9xnonsmoker	11 x non-smoker
Months since diagnosis	0 (0 - 0)	210 (97 - 310)	72 (8-1123)
Concomitant drugs	12 x nil	12 x nil	11 x nil
normal colonoscopy	12/12	0/12	0/11
Left sided colitis	0/0	4/12	6/11
total colitis	0/0	8/12	5/11
Baron score	0 (0–0)	0 (0–0)	3 (3-3)
Mayo Score	0.0 (0–5)	0.0 (0–2)	9 (6–12)
Hb	129 (125 – 144)	140 (138 - 143)	136 (110-145)
ESR	6 (3 – 15)	18 (4 – 15)	40 (15-98)
CRP	3 (0 – 11)	5 (2 – 8)	19 (8-33)

Table 27. Demographic of patients from which mucosal biopsies were taken and TSLP quantified by ELISA

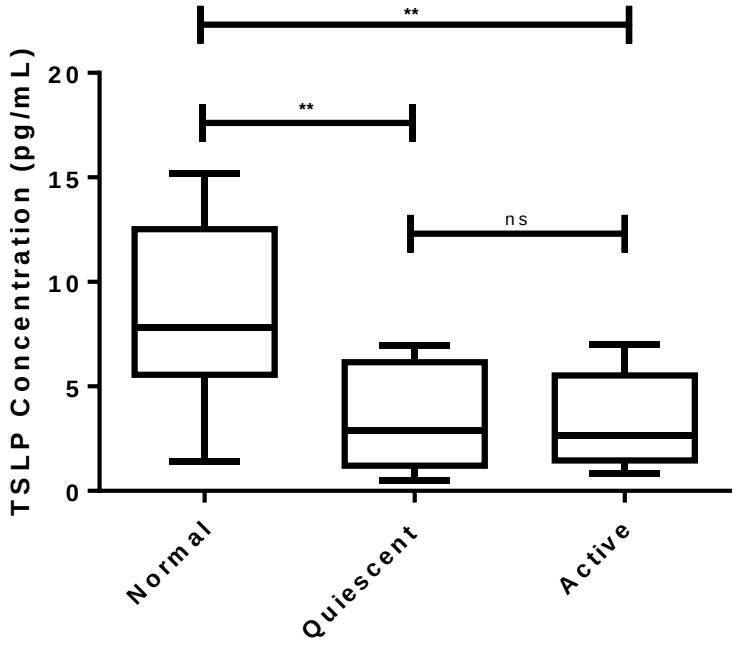


Figure 74. TSLP protein concentration analysed by ELISA, in the sigmoid mucosa of active and inactive UC, and in healthy controls

In all three groups the protein concentration was low. In healthy controls the TSLP protein concentration is 7.8 pg / mL (IQR 5.6 – 12.5). In inactive UC the median protein concentration is 2.9 pg / mL (IQR 1.2 – 6.2), and in active UC the median protein concentration is 2.6 pg / mL

3.4.5 TSLP mRNA expression before and after Tralokinumab exposure in moderate to severe UC

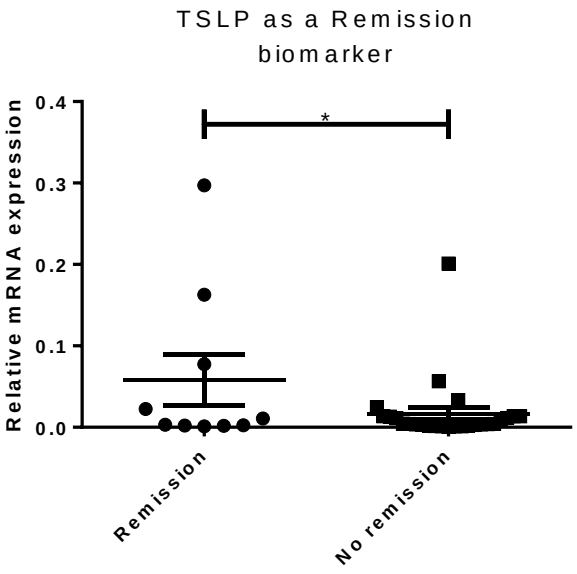


Figure 75. The levels of TSLP in inflamed tissue can possibly predict patients who will achieve Remission with Tralokinumab

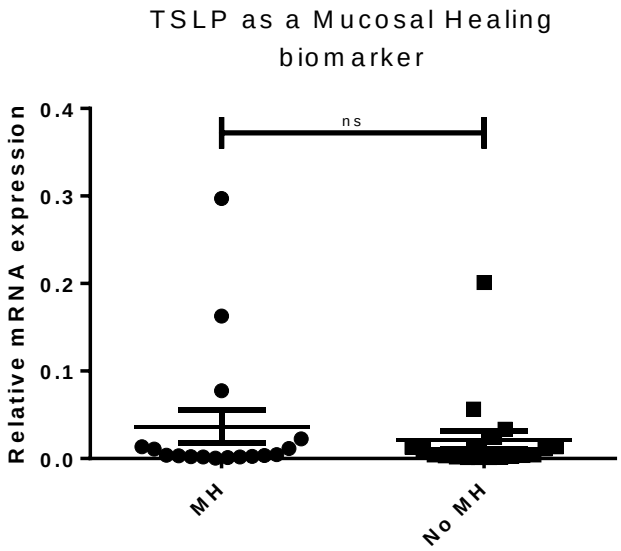


Figure 76. The levels of TSLP in inflamed tissue cannot predict who will develop Mucosal Healing with Tralokinumab

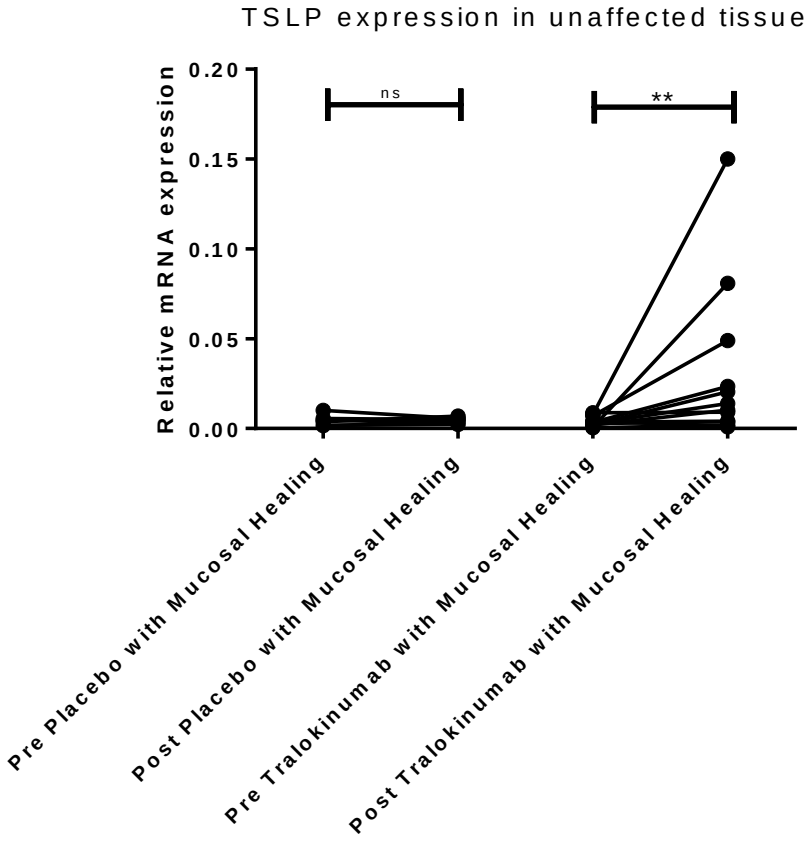


Figure 77. The levels of TSLP mRNA expression are increased when mucosal healing is achieved but only in those patients who have achieved this with tralokinumab therapy

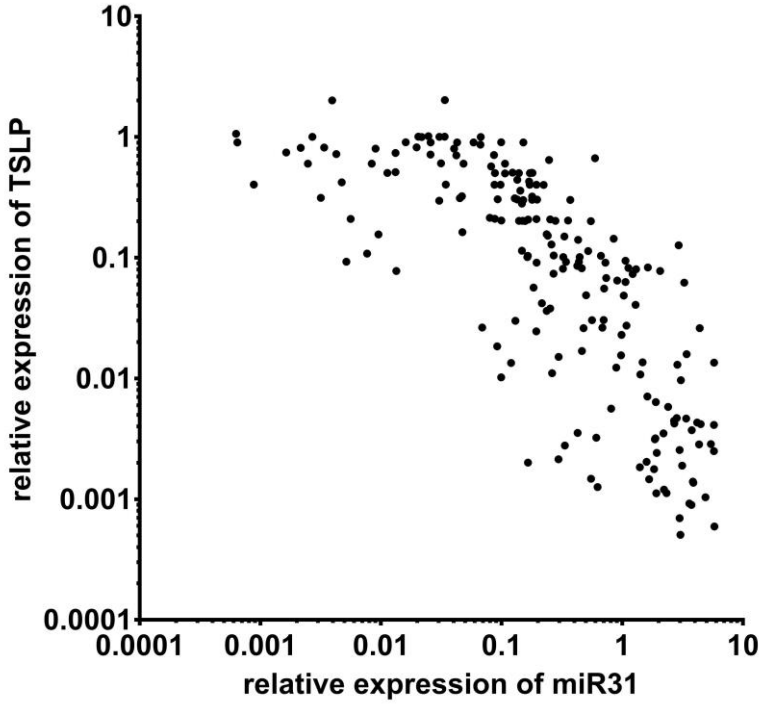


Figure 78. Regression curve showing the correlation between TSLP expression and miR-31 expression

Unlike miR-31 TSLP cannot predict mucosal healing as a response to Tralokinumab treatment, however there is a suggestion that it could predict clinical remission. Interesting TSLP mRNA expression is only upregulated in the presence of mucosal healing when it is achieved by treatment with Tralokinumab, and not in the group of patients who achieve mucosal healing spontaneously i.e. with placebo. This suggests not only a specific mode of action for the development of mucosal healing with Tralokinumab, but also that these patients may develop a sustained response to Tralokinumab, and that its role may be more beneficial as a maintenance of remission therapy rather than an inducer of remission.

3.5 MiR-31 targeting of TSLP 3'UTR

Mir-31 has been identified as a potential target for the 3'UTR of TSLP on both bioinformatics searches and in the previous chapter results showing that upregulated expression of miR-31 is associated with both reduced expression of TSLP mRNA, and reduced mucosal concentration of TSLP protein in active UC.

The targeting of TSLP 3'UTR by miR-31 was analysed co-transfecting wild type or mutated miR-31 binding site reporter constructs with miR-31 over expressing vector in HeLa cells and employing Dual Luciferase Assay.

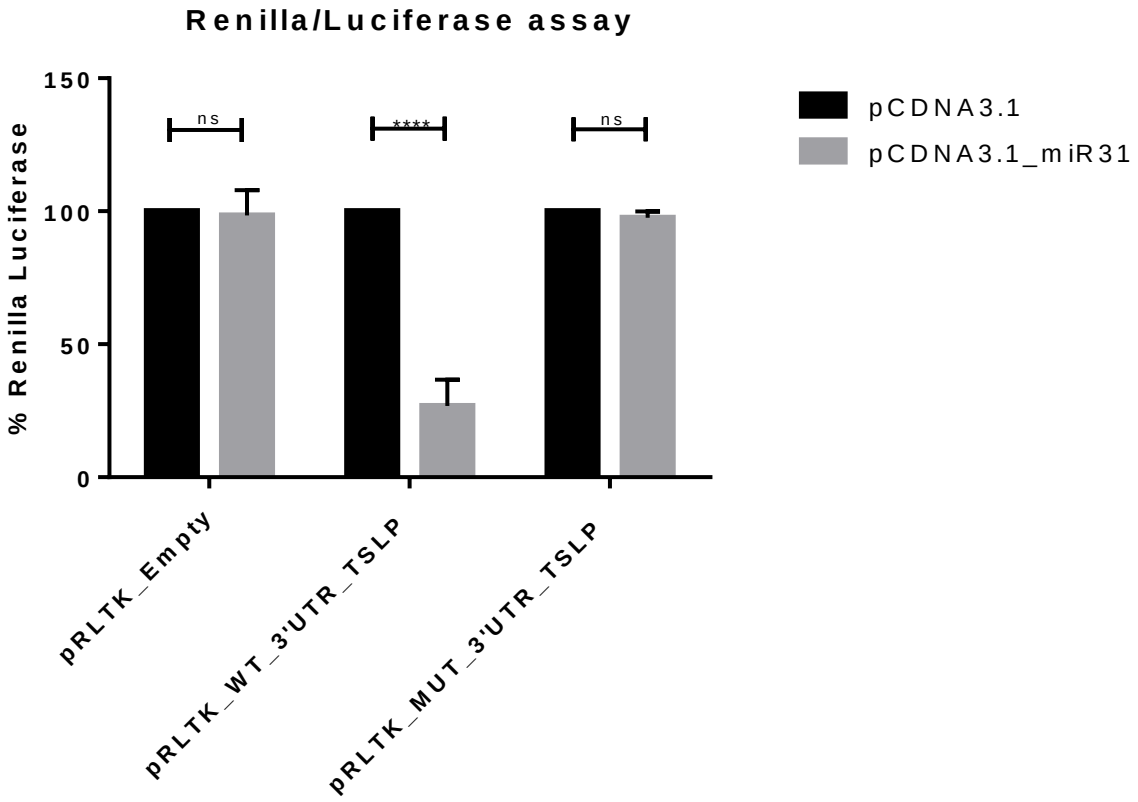


Figure 79. Renilla/Luciferase assay showing the effect of miR-31 on gene expression by targeting 3'UTR_TSLP

Figure 79 showed that co transfection of miR-31 with the wild type construct led to a significant reduction of the renilla luciferase activity by 70%, in 3 independent experiments. Mutation of the predicted binding site for miR-31 reversed these effects. This demonstrates that miR-31 directly binds to the 3'UTR of TSLP.

This also provides the explanation for the link between TSLP and miR-31 expression seen in Figure 78.

4. Overall Discussion

This data shows that specific cytokine and microRNA changes may be due to medication. This has been replicated in an ex vivo model. The three disease states of normal, inactive and active have a specific profile of expression and can be distinguished from one another as such. Crohn's colitis interestingly follows a more similar profile to UC, showing characteristics of Th2 type inflammatory response. The treatments that are used in IBD all have different effects on the expression of these microRNA and cytokines. Interestingly Adalimumab and Infliximab suggest some different effects on the expression profiles in the three disease states. This could be due to the molecular structure of these anti-TNF monoclonal antibodies differing in such a way as to change the penetration into the tissue or pathways by which they act to reduce inflammatory response.

By replicating the disease treatments and expression profile changes in the ex-vivo model, I have shown that the response of the microRNA and cytokine expression to treatment, maybe due to the specific treatment being administered and not due to factors such as disease severity, or duration of treatment.

By showing this can be done in an ex-vivo model, this model could be utilised clinically, by using the profiles as biomarkers to predict disease response to specific treatments. This way individual patients could receive personalised treatment strategies, hopefully improving outcomes by treating with the correct therapy early, and avoiding side effects and complications of therapy that does not benefit the individual patient.

Also, by doing this the healthcare system may save money by not exposing patients to treatments that they are predicted to have a limited benefit to whilst possibly experiencing significant side effects.

The expression profiles may also aid with diagnostic pathways in patients whose colitis is unclassified, thus informing on future prognosis and treatment strategies.

This model does however have several weaknesses which may affect the results seen and could mean the model is not as reliable in replicating what is happening in reality. The thiopurine used in this model is the active metabolite of the drug Azathioprine, in the form of 6-thioguanine. Clinically when azathioprine is used it therapeutic effect can take several

weeks to peak, however in this model I was measuring the microRNA and cytokine profiles at 24 hours.

The concentration of the drug in the media in which the biopsies has been estimated using other research and I cannot be sure that the tissue concentrations of each drug accurately reflect that of tissue in vivo.

Finally, these drugs exert their effect on immune cells both within the target tissue but also in immune cells being recruited from the circulating immune system, however in this model recruitment of circulating immune cells will not happen as we are dealing with an ex vivo treatment of a mucosal biopsy. Thus, the mechanism of action by which in the immunological mediating drugs work, will not be reflected accurately in this model.

Clearly there would need to be more investigation in to this model, by treating the patients alongside the ex vivo treatment of the biopsies to see if there is a correlation between in vivo and ex vivo cytokine and microRNA profiles before it could be used clinically.

4.1 Ulcerative Colitis

I showed that in active UC miR-31 expression was decreased in the mucosa of patients treated with Azathioprine however this observed decrease in expression was not seen in Adalimumab treatment, and was less well observed in Infliximab treatment. These samples were however unpaired, and the length of time these patients had been on each drug and the initial severity was variable. Despite these differences the effects treatments had on miR-31 expression that was seen in the in vivo experiment was preserved in the ex vivo experiment with paired samples when these factors were eliminated, when comparing 6-TG treatment to biological therapies as seen in Figure 80.

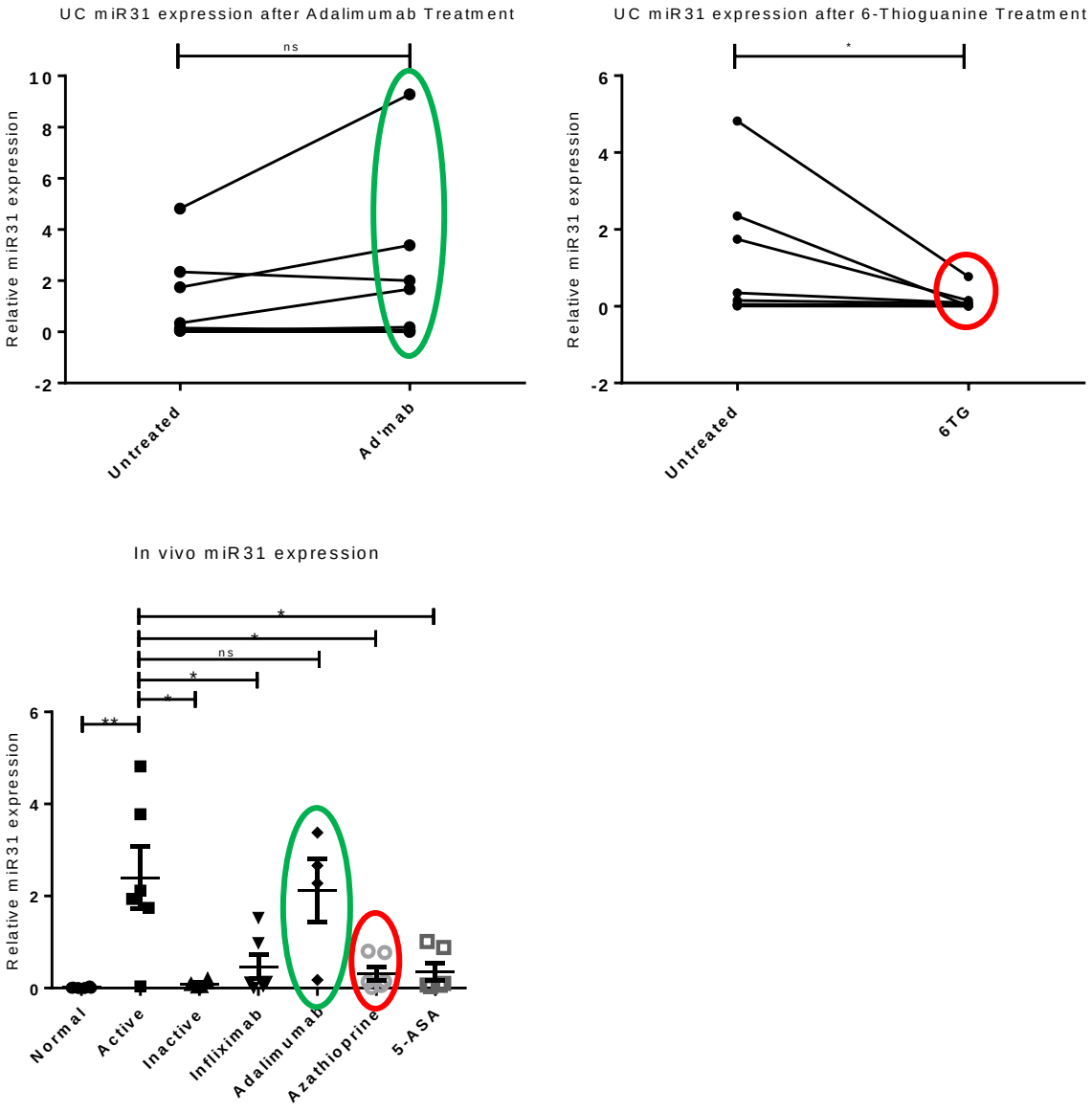


Figure 80. Comparison of Figures 3 and 22, the results of how drugs effect miR-31 expression in UC in vivo and in the ex vivo experiment

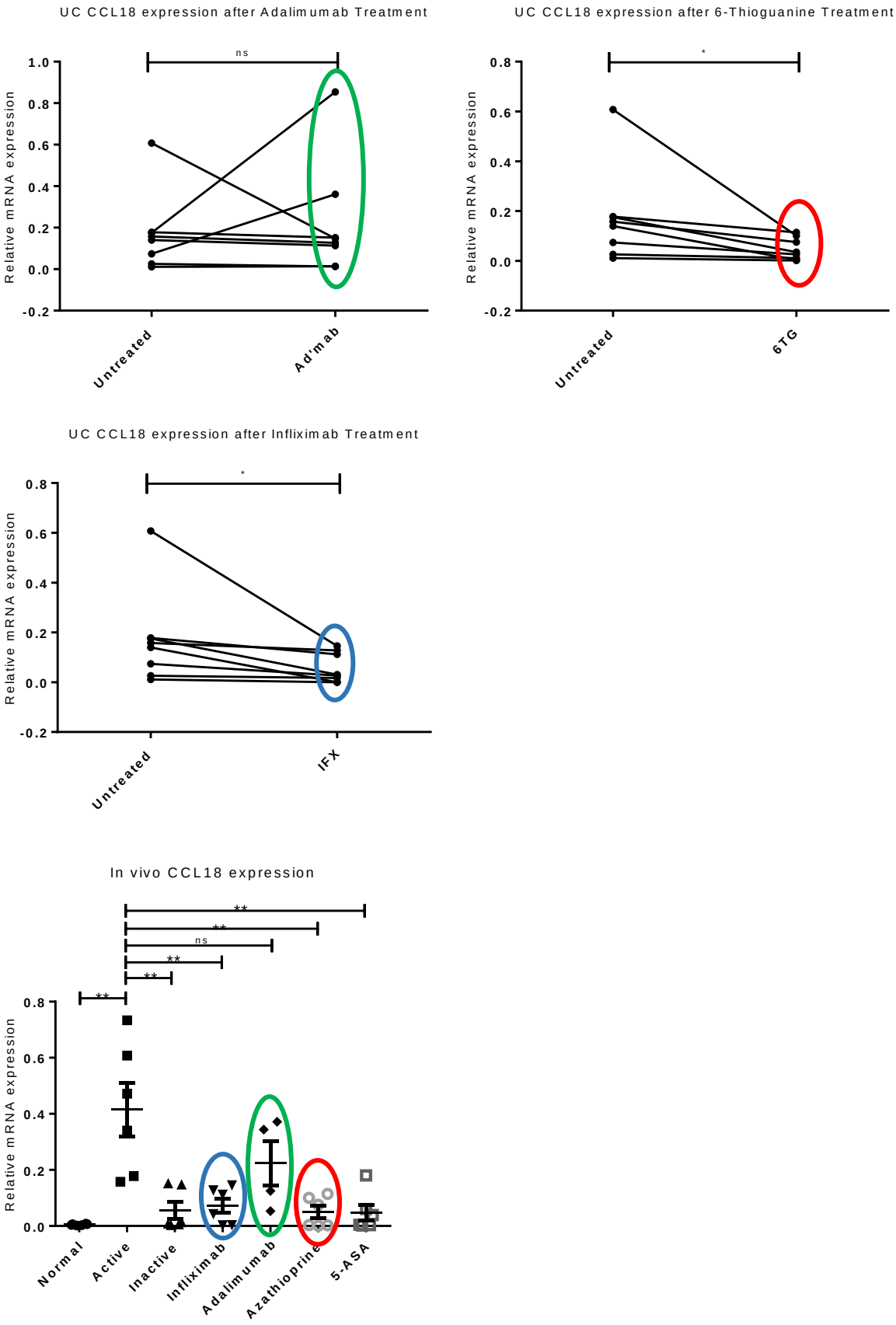


Figure 81. Comparison of Figures 6 and 25, the results of how drugs effect CCL18 expression in UC in vivo and in the ex vivo experiment

Therefore, there appears to be a specific mode of action of Azathioprine that reduced the expression of miR-31 in the colonic mucosa of active UC, which does not appear to happen with biological therapies.

CCL18 is an indirect target of miR-31, and the same pattern of expression is seen when looking at patients on Azathioprine and Adalimumab, and again this is replicated in paired samples of active UC treated in an ex vivo experiment. However, in this case infliximab does reduce the expression of CCL18 in the colonic mucosa as seen in Figure 81. The reason behind the difference seen in the expression of miR-31 and CCL18 between Adalimumab and Infliximab cannot be established, however although they are both anti-TNF therapies, Infliximab is a chimeric monoclonal antibody whereas Adalimumab is fully humanised which may suggest different modes of action in terms of the modulation of cytokine expression. Alternatively, it could be that experimentally the dose used of Adalimumab was inadequate to achieve the same response, and as this is also seen in vivo this could be adding weight to the idea Adalimumab is either less effective in UC or that there needs to be improved dose optimisation in these patients.

The replication seen in the Ex Vivo setting is not just confined to miR-31 and CCL18. Figure 82 shows how the effects of miR-146a expression by Azathioprine treatment is to reduce its expression in vivo, and that this reduced expression is also seen in the ex vivo setting. Infliximab as an example does not have this effect of the expression of miR-146a when used as therapy in vivo, and the ex vivo study also shows no significant change in miR-146a expression after the explants have been treated with infliximab.

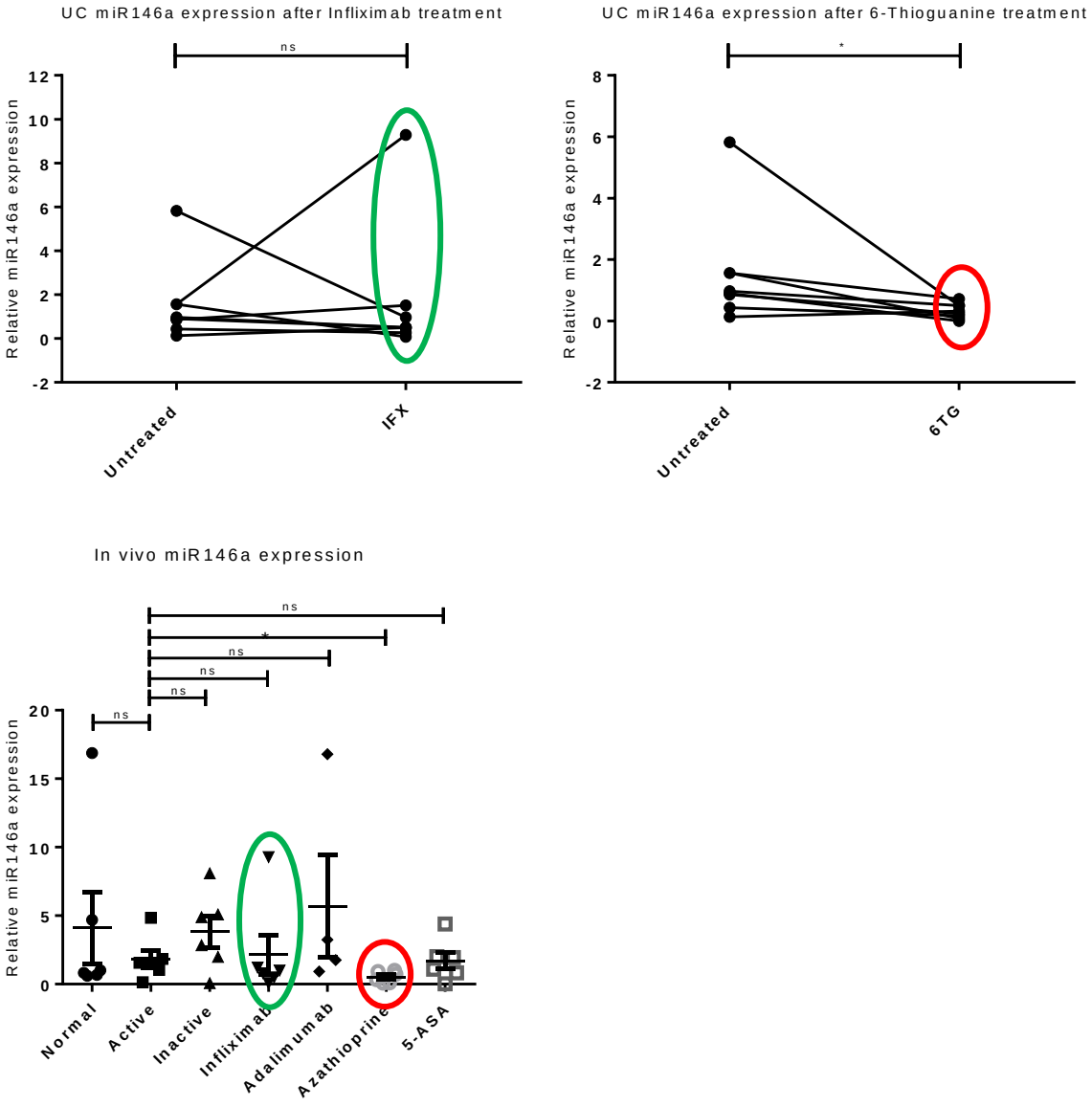


Figure 82. Comparison of Figures 4 and 23, the results of how drugs effect miR-146a expression in UC in vivo and in the ex vivo experiment

Again, Figures 83 and 84, miR-155 and TNFα expression alterations seen in vivo in the mucosa of active UC on different therapies, are able to be replicated in an ex vivo model, again validating that this model could be representative of the in vivo pathways .

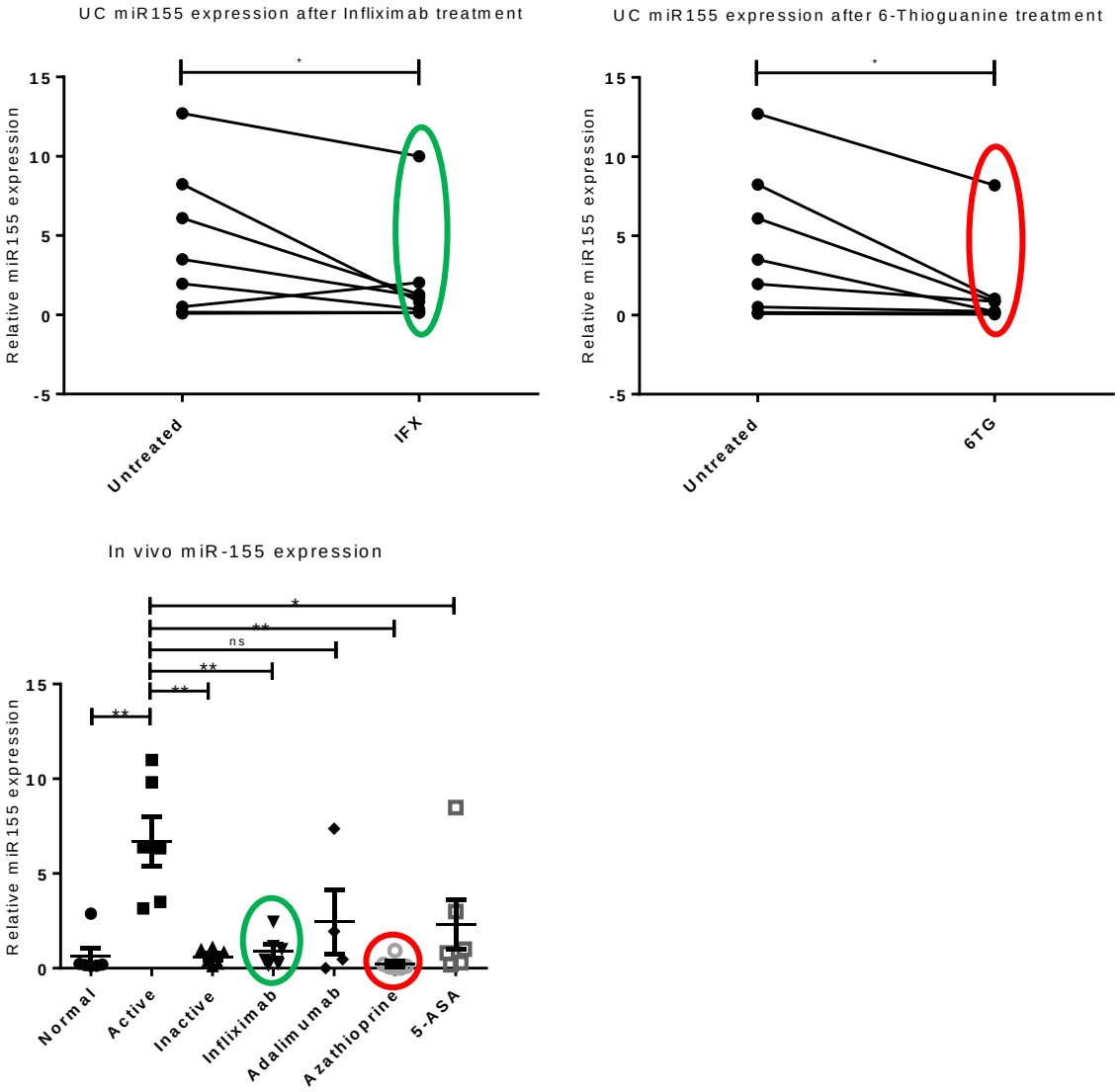


Figure 83. Comparison of Figures 5 and 24, the results of how drugs effect miR-155 expression in UC in vivo and in the ex vivo experiment

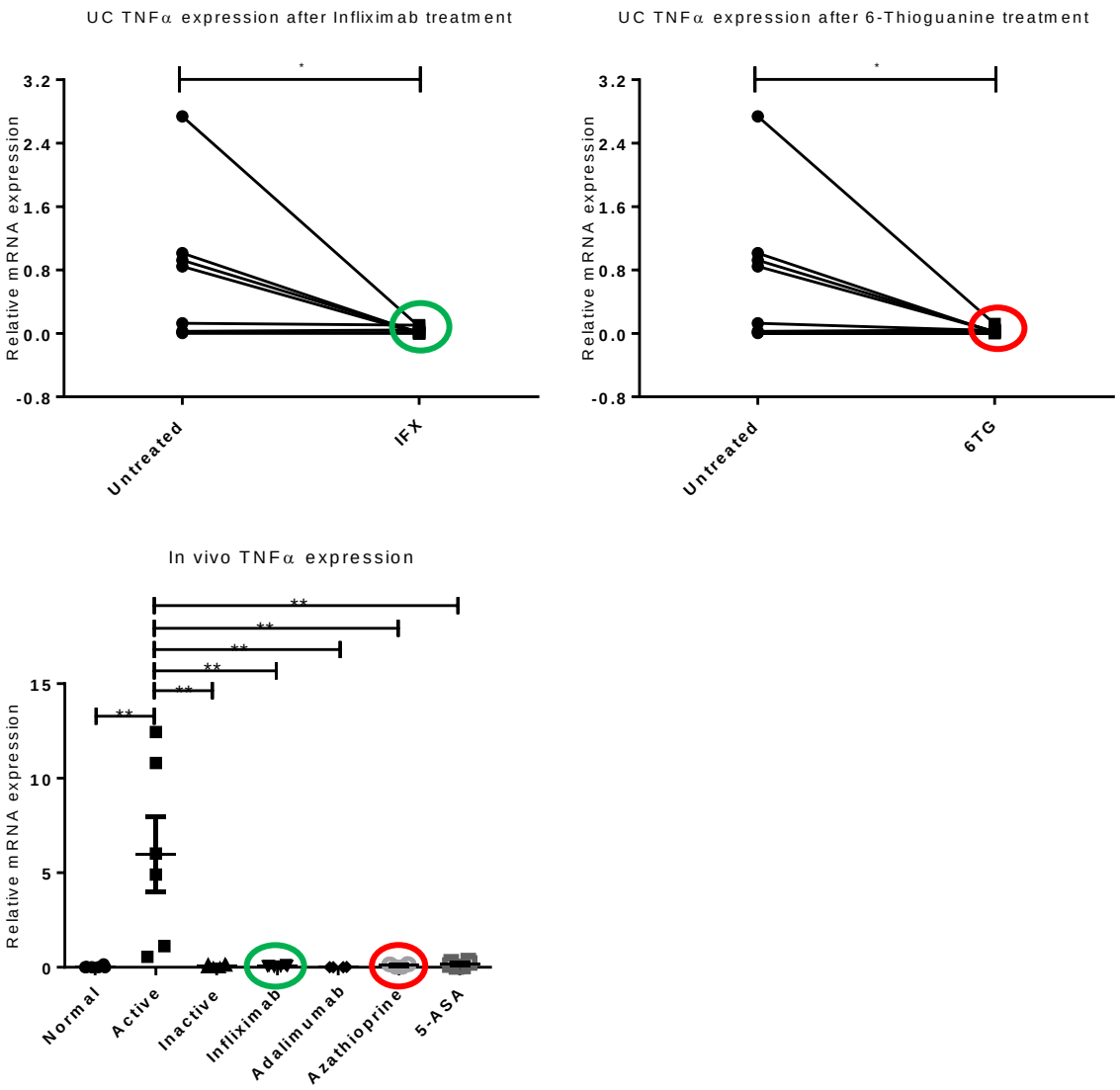


Figure 84. Comparison of Figures 8 and 27, the results of how drugs effect TNFα expression in UC in vivo and in the ex vivo experiment

The consistent replication of gene and miRNA expression seen in the ex vivo setting when compared to the in vivo experiment may imply that the ex vivo experimentation of treatment naïve mucosa could be a useful tool to predict treatment response. The Figures show that there is differential gene and miRNA expression between these treatments, especially miR-31 and CCL18, although looking at the samples individually not all of them did respond in the same way. It could be hypothesised that these patients will not respond to these treatments, but would do better on alternative therapy. A study comparing ex vivo expression change and clinical and endoscopic disease response to therapy would be required.

In our analysis, miR-31 showed potential as a biomarker to predict whether clinical response or mucosal healing could be achieved in response to Tralokinumab therapy. If patients with raised miR31 were excluded from the analysis, clinical response rates increased from 47% to 52%, mucosal healing rates from 44% to 57%, and clinical remission from 26% to 32%. When applying the same corrections on the placebo arm of the study, there was no significant increase in rates of these responses.

miR-31 expression was significantly down regulated in those patients who achieved mucosal healing with Tralokinumab but not patients who achieved this in the placebo group. Thus, this down regulation was independent of mucosal healing as it was not seen in those patients with spontaneous mucosal healing (placebo). The importance of miR-31 downregulation after Tralokinumab treatment may be underestimated as some of those patients treated with Tralokinumab may have achieved spontaneous healing despite the treatment (and thus have shown improvement without miR31 reduction). The length of remission achieved in placebo patients who did not reduce miR31 levels would have been interesting to follow up for longer than the study design permitted, as the miR31 levels maybe be time dependant and, in some patients, have not been reduced to the full potential.

This particular result offers insights into the mechanisms of action of Tralokinumab in UC which seems to directly affect the IL-13 mediated inflammation of the gut, as reflected by the levels of miR31, a microRNA linked with IL-13 inflammation(*198, 208*).

miR31 may give an insight into why Tralokinumab did not have a higher response rates in a study designed to assess its efficacy as an induction agent as opposed to a maintenance agent. Our data shows that patients with high expression of miR31 responded poorly to Tralokinumab. We have demonstrated possible evidence of IL-13 mediated inflammation in active ulcerative colitis, but not the presence of the therapeutic target of Tralokinumab in the gut mucosa at the point in time that the drug was administered. IL-13 expression may be upregulated early in the disease process by an unknown trigger, initiating mucosal damage and activating Th2 inflammatory pathways. Increased miR31 expression may reflect the later stages of an IL-13 induced inflammatory process, where IL-13 has been down regulated by miR-31 as part of a negative feedback loop, thus removing the therapeutic target for Tralokinumab. miR-31 targets IL13Rα1 and this reduces sensitivity to IL-13(*208*),

but bioinformatics suggests that miR-31 may also reduce IL-13 expression as well. This hypothesis is strengthened by the difficulties in actually measurement of IL-13 either by ELISA or mRNA expression by RTqPCR in inflamed tissue(*208*). Therefore, patients would not benefit from an anti-IL-13 drug as an induction agent as the therapeutic target has been down-regulated soon after the initial trigger event for the flare, perhaps even before the patient becomes symptomatic. On this basis it is possible that anti-IL13 drugs may be potent maintenance agents rather than induction agents. The design of the current trial does not allow this hypothesis to be further explored.

Little is known of the role of TSLP in the intestine. Although current evidence suggests that it is expressed by epithelial cells at the barrier surface in response to the luminal flora and/or invasive pathogens. In vitro studies have also demonstrated TSLP expression is also driven by TLR ligands, viruses and a variety of cytokines including TNF-α and IL-4(*217*).

We show here a clear relationship between miR31 and TSLP by the direct targeting and downregulation of TSLP by mir31. TSLP is clearly increased in the mucosa during remission and most importantly, it significantly increases in most of the patients that achieved mucosal healing with Tralokinumab treatment and not with placebo. This result concurs with the fact that miR-31 is decreased after treatment in the same group. Furthermore, in Jurkat cells cultured with the antagomir of miR-31 not only is the increase in miR-31 negated in stimulated cells but also the effect on TSLP is prevented, indicating an effect of miR-31 on the TSLP pathway.

There is no doubt that UC is a complex disease that has failed to be explained by a single unifying theory. This is reflected in the difficulty in diagnosis, management and outcomes. The finding of a decreased TSLP expression in UC is important because it links together and provides a mechanism for several disordered features of UC such as the disordered response to luminal antigens, and the breakdown of regulation of the epithelial barrier. Understanding the role of TSLP in acquired immunity and regulation of T helper cell responses is not only diagnostically helpful but could also provide possible therapeutic options.

4.2 Crohn's Disease

Colonic Crohn's disease, unlike ileal Crohn's disease, has similar miR-31 and CCL18 expression, to UC. As you can see from Figure 83. Azathioprine has a greater effect at lowering miR-31 and CCL18 expression than anti-TNF therapies do. However, the anti-TNF therapies are much better at lowering CCL18 expression in the colonic mucosa of active Crohn's disease than UC. Whether this translates into improved clinical response cannot be said, but it could suggest that anti-TNF therapies may be more efficacious in colonic Crohn's disease than in UC.

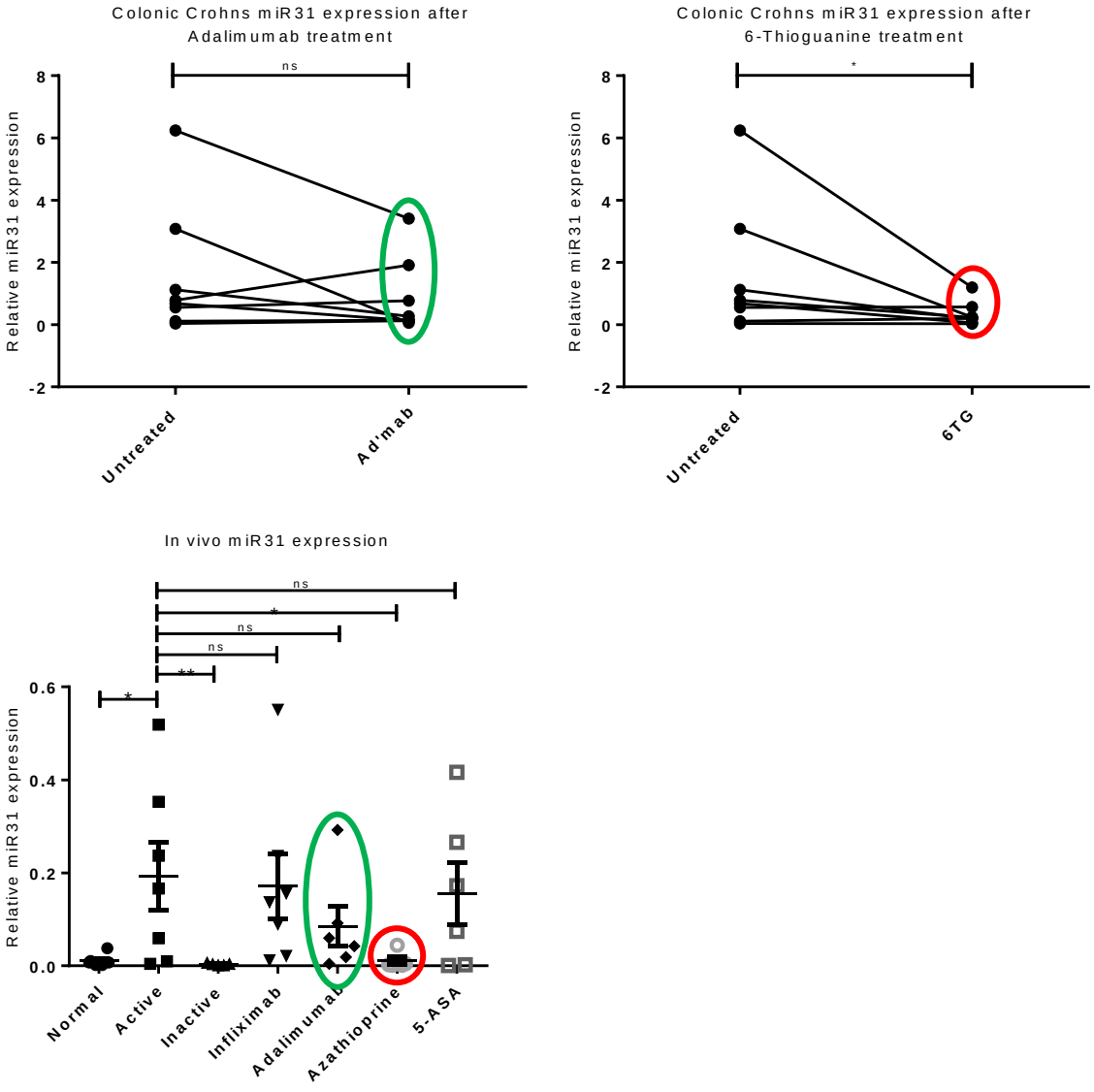


Figure 85. Comparison of Figures 9 and 29, the results of how drugs effect miR-31 expression in Colonic CD in vivo and in the ex vivo experiment

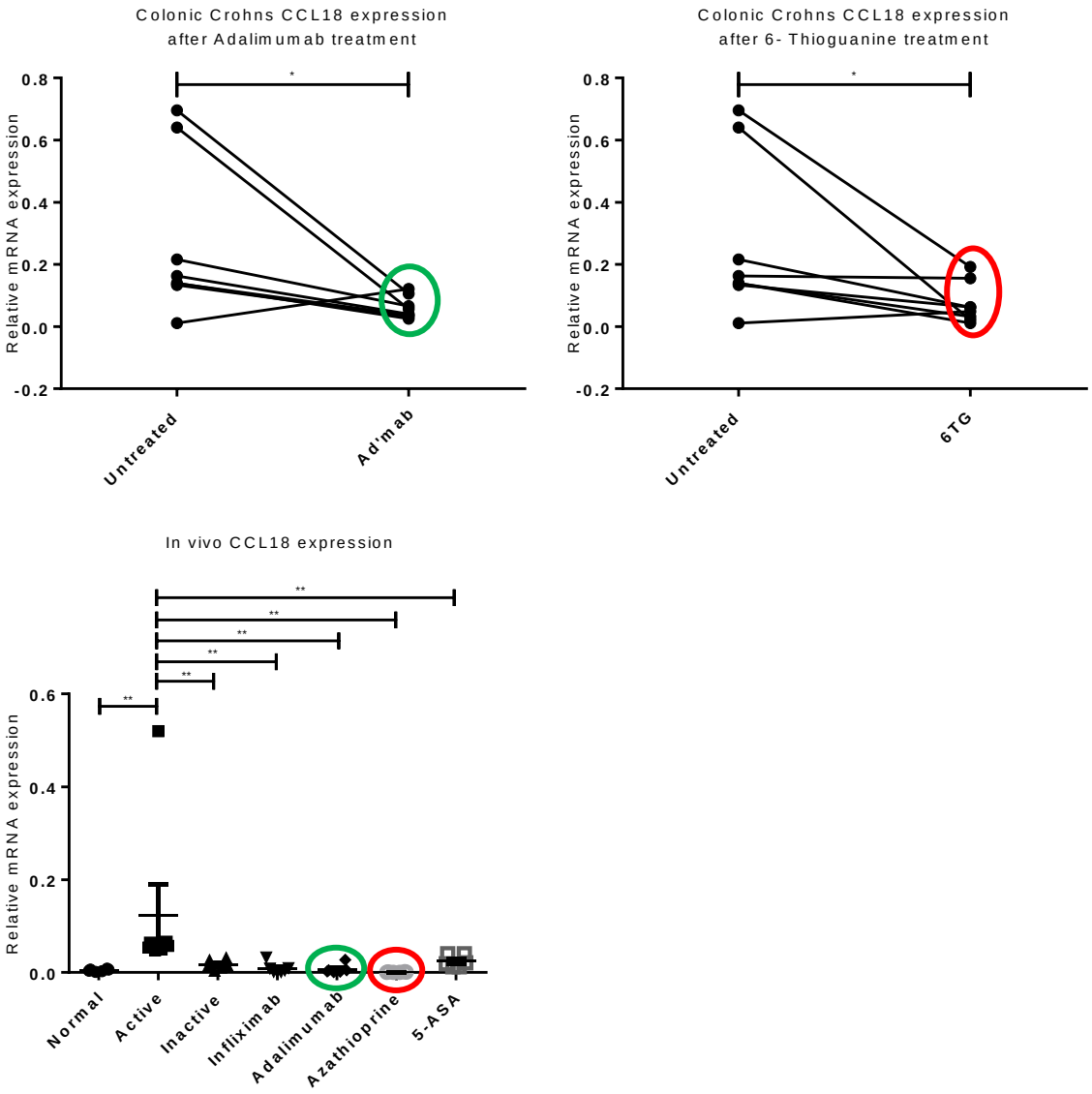


Figure 86. Comparison of Figures 12 and 32, the results of how drugs effect CCL18 expression in Colonic CD in vivo and in the ex vivo experiment

Similarly, to UC, colonic Crohn's disease mucosal miR-146a expression is lowered by Azathioprine treatment, and 6-TG treatment in the ex vivo model. This response is again not seen in the anti-TNF treatments groups, or in ex vivo treatment of the mucosal biopsies. The difference seen between UC and colonic Crohn's is that there is an over expression of miR-146a in the colonic mucosa of active colonic Crohn's, when compared to both inactive colonic Crohn's disease and normal colonic mucosa. This could not be elucidated in the mucosal biopsies of UC.

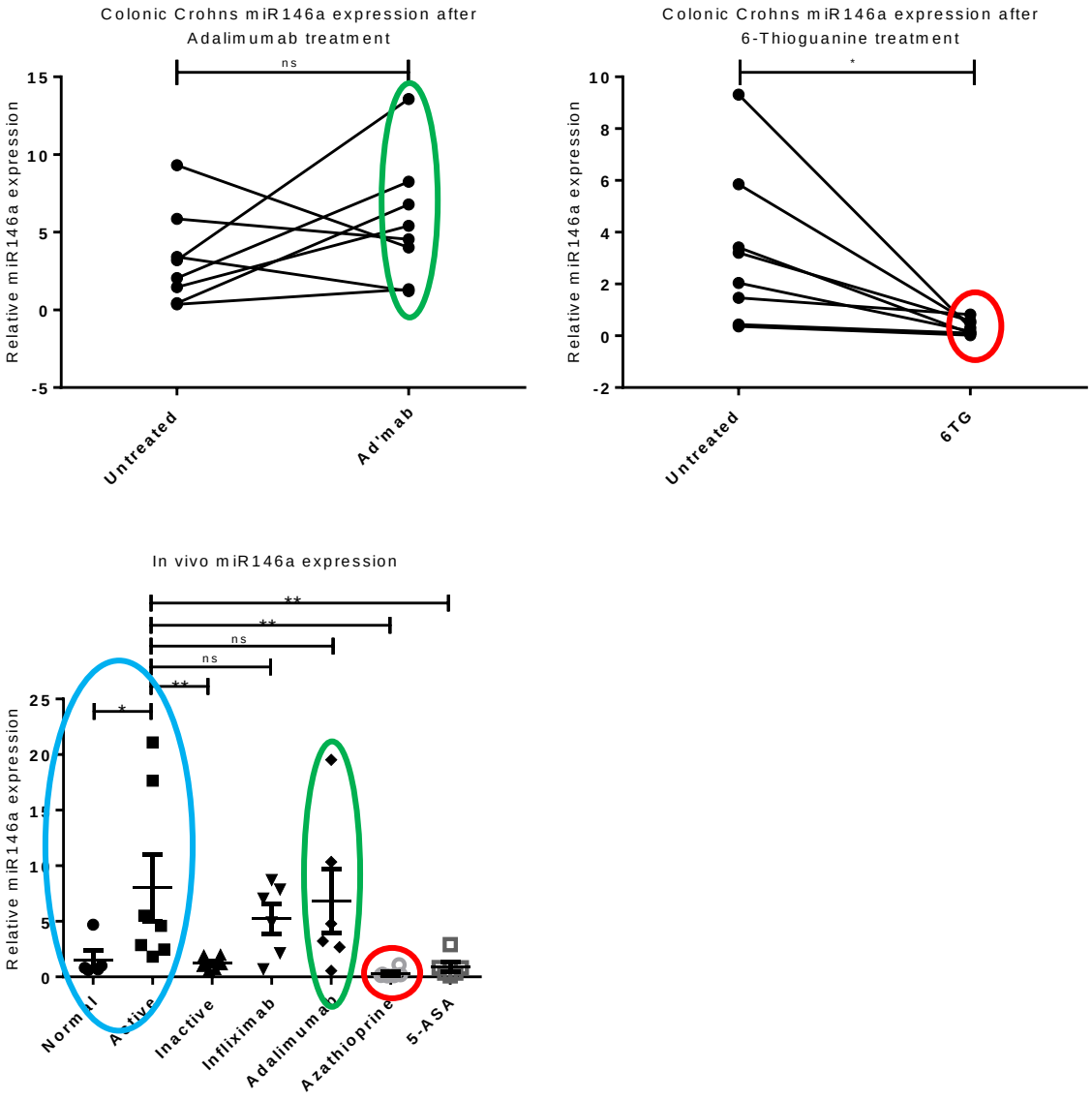


Figure 87. Comparison of Figures 10 and 30, the results of how drugs effect miR-146a expression in Colonic CD in vivo and in the ex vivo experiment

Crohn's disease of the ileum has an entirely different pattern of microRNA and cytokine expression when compared to colonic disease. miR-31 and CCL18 expression both have greater expression in normal ileal mucosa than in normal colonic mucosa. CCL18 expression is also down regulated in active and inactive ileal disease, and up regulated in colonic disease when compared to normal ileal and colonic mucosa. Although not significant, in ileal disease, this trend for down regulation appears with miR-31 expression as well.

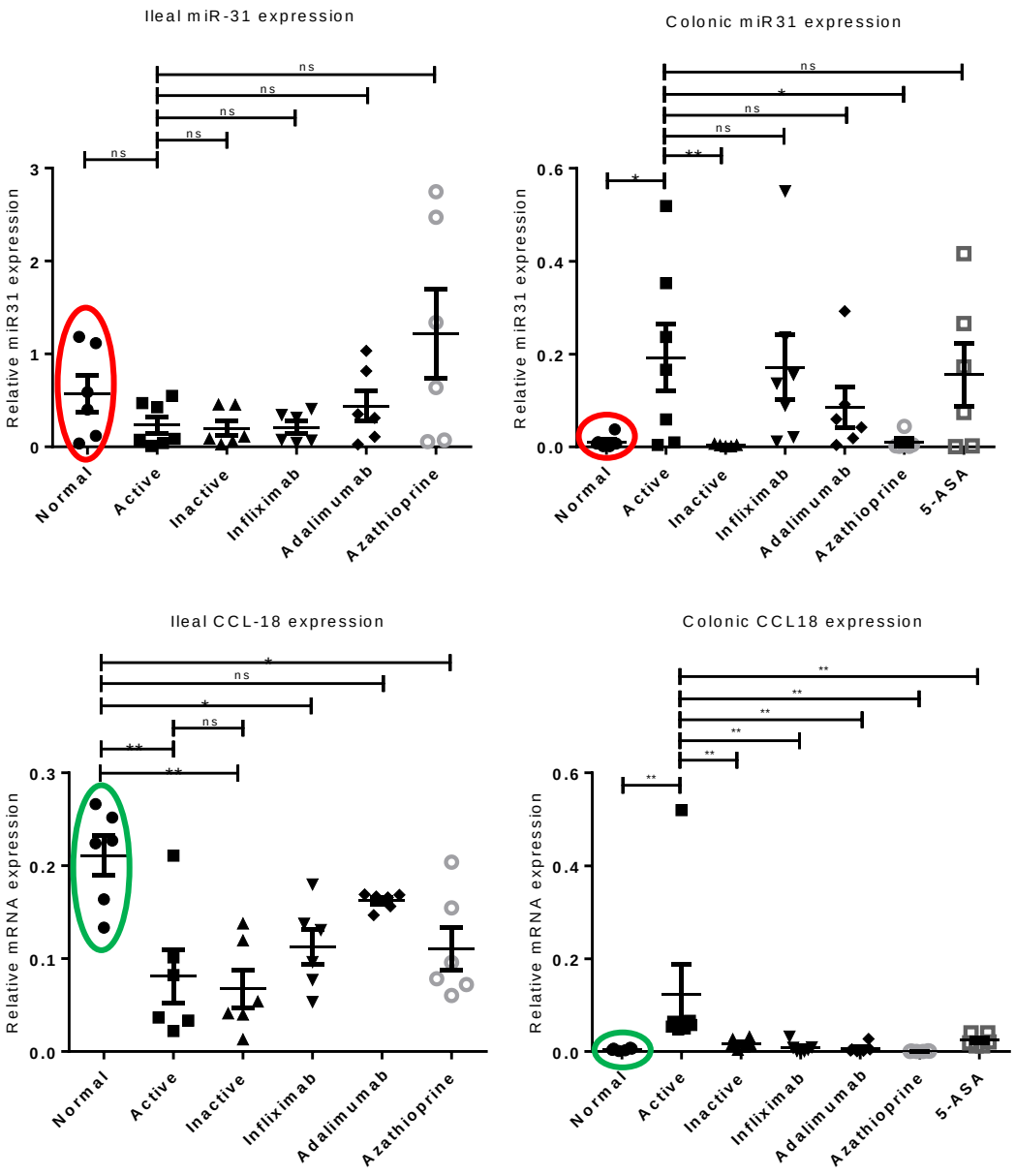


Figure 88. Comparison of Figures 9, 12, 15 and 18, the results of how drugs effect miR31 and CCL18 expression in Colonic and ileal CD in vivo

Also, the response that occurs in relation to miR-31 and CCL18 expression and drug treatment is also very different. Ileal disease does not have significant change across the treatment groups and this reflected in the ex vivo model as shown in Figure 87. However, in contrast to colonic disease in the in vivo study, Azathioprine does show a trend to increase miR-31 expression in ileal disease. It is possible that either azathioprine has a different mechanism of action in the ileum, or that azathioprine is of less benefit in ileal disease.

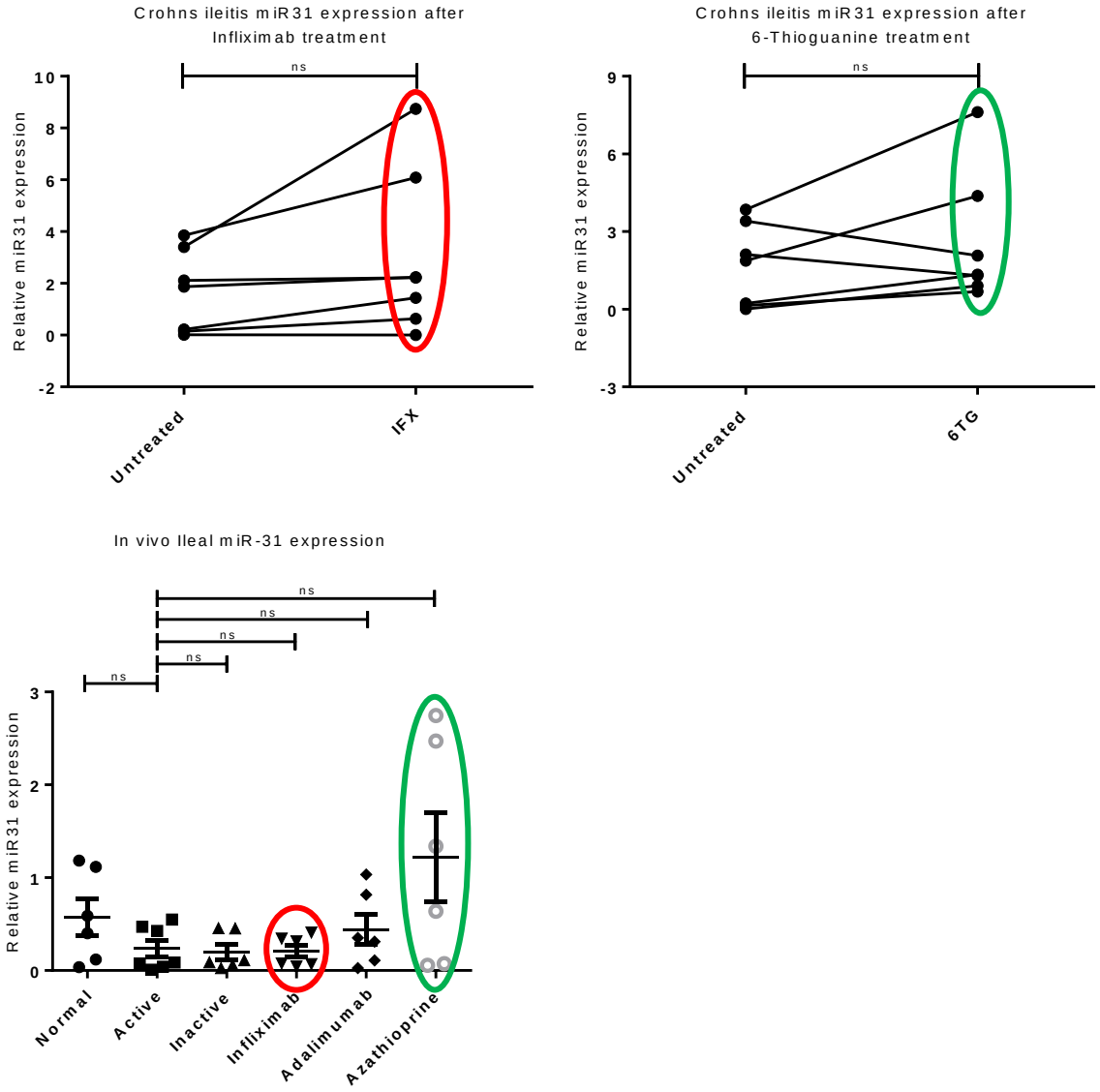


Figure 89. Comparison of Figures 15 and 36, the results of how drugs effect miR-31 expression in Ileal CD in vivo and in the ex vivo experiment

I have used 5-ASA therapy as an ex vivo treatment of both Crohn's disease and ulcerative colitis. Clinical trials have now shown that there is little value in the use of 5-ASA therapy in Crohn's disease. The ex vivo data has indeed shown that in the cytokine and microRNA

profiles I have studied that 5-ASA therapy has no significant effect in actively inflamed small bowel mucosa. There was, however, statistically significant reduction in these profiles in the mucosa of colonic Crohn's disease treated with 5-ASA therapy. This could be that 5-ASA therapy does play a role in the treatment of colonic Crohn's disease although trial data does not support this, or that these cytokines are not a great reflection of the activity of Crohn's disease in the colonic mucosa when the disease has been treated.

The final table summarises the change in expression of the measured cytokines and microRNAs in the mucosa in each active disease state when compared to normal mucosa.

	Ulcerative Colitis	Crohn's Colitis	Ileal Crohn's disease
miR-31	↑	↑	↓
miR-155	↑	↑	↑
miR-146a	↔	↑	↔
CCL18	↑	↑	↓
SOCS1	↔	↔	↔
TNFα	↑	↑	↑

Table 28. Table showing the general change in expression of the measured cytokines and microRNAs in each disease state.

5. Future Work

Further work is needed to discern the target genes and their function to better understand the pathological effect of miRNAs. MiRNAs are a promising field for development of biomarkers in both IBD and other diseases. Research is needed to develop this area and validate their use as biomarkers for diagnosis and disease activity. My work may suggest that a carefully selected panel of microRNAs could be a useful biomarker for predicting disease response to conventional therapy, and also novel therapies. Future studies involving analysis of RNA expression in tissue from patients' pre and post novel therapy may help predict whether these new treatments will have a specific target group if broad use of the drug is not efficacious.

The ex vivo model may be a useful technique to predict disease response, but this would need to be validated in a clinical trial. The real value of this model is probably limited as several variables which I have not controlled for would need to be taken into account for it to be validated. Timing and dosing of the drugs would need to be examined further, possible using immunohistochemistry or in-vivo tissue staining techniques to quantify tissue drug concentrations. Also standardising the makeup of biopsies using flow cytometry and cell sorting would be useful. However, using all these techniques would likely make the model time consuming and expensive, and therefore not practical in clinical practice.

MiRNAs are now being research as targets for drug therapy in other diseases such as malignancy and HCV. This gives promise to the idea that miRNA could be targeted therapeutically in the future, but also improved understanding of which pathways miRNA regulate could reveal new drug targets. This could involve direct anti-microRNA therapy, or miRNAs themselves to inhibit genes known to cause inflammation.

Using RNA sequencing, to assess predominant expression of RNA and microRNA in individual patients, genomic targeting of patents with specific drugs may aid the treatment of patients, to give specific personalised medical therapies.

I have shown that miR-31 regulates TSLP mRNA and protein expression, however this pathway alone may not be sufficient to influence the immune response and disease phenotype in individuals. This mechanism should be further investigated to see if

manipulation of this process could offer new insights in to potential targets for novel therapy.

Finally, I have hypothesised that although Tralokinumab was unsuccessful in reaching the primary outcomes for induction of remission of moderate to severe UC, by extending the trial to a longer course of therapy Tralokinumab may prove efficacious as a drug used in the maintenance of remission. A study design like this would require a much longer follow period however and may not prove cost effective for the drug company.

6. Publications and Abstracts

Whiteoak SR, Felwick R, Sanchez-Elsner T, Fraser Cummings JR. MicroRNAs in Inflammatory Bowel Diseases: Paradoxes and Possibilities. *Inflamm Bowel Dis.* May 2015;21(5):1160-1165.

Simon Whiteoak, Tilman Sanchez-Elsner, JR Fraser Cummings. Drug therapies in Ulcerative Colitis influence the expression of microRNAs and cytokines in the sigmoid mucosa in an ex vivo model. *Gastroenterology.* Volume 148, Issue 4, Supplement 1, Pages S-695, Digestive Disease Week, Poster Presentation, Washington DC, USA, May 2015

Simon Whiteoak, Tilman Sanchez-Elsner, JR Fraser Cummings. Cytokine and microRNA expression in colonic and ileal Crohn's disease is modified by drug therapy in an ex vivo model. *Gastroenterology.* Volume 148, Issue 4, Supplement 1, Pages S-695 – S-696, Digestive Disease Week, Poster Presentation, Washington DC, USA, May 2015

Simon Whiteoak, Tilman Sanchez-Elsner, JR Fraser Cummings. Drug therapies in Ulcerative Colitis influence the expression of microRNAs and cytokines in the sigmoid mucosa in an ex vivo model. *Gut,* 2015;**64**:Suppl 1 A438, British Society of Gastroenterology, Poster Presentation, London ExCel, June 2015

Simon Whiteoak, Tilman Sanchez-Elsner, JR Fraser Cummings. Cytokine and microRNA expression in colonic and ileal Crohn's disease is modified by drug therapy in an ex vivo model. *Gut,* 2015;**64**:Suppl 1 A438-A439, British Society of Gastroenterology, Poster of Distinction, London ExCel, June 2015

Simon Whiteoak, Andrew Claridge, Tilman Sanchez-Elsner, JR Fraser Cummings, Targeting of TSLP by miR-31 may play an important role in mucosal healing in Ulcerative Colitis. *Journ Crohn's Colitis,* (2016) 10 (suppl 1): S92-S93. European Crohns and Colitis Organisation, 11th Congress, Amsterdam, 16-19 March 2016

Simon Whiteoak, Andrew Claridge, Tilman Sanchez-Elsner, JR Fraser Cummings. Targeting of TSLP by miR-31 may play an important role in mucosal healing in Ulcerative Colitis. Awaiting publication

Markus Gwiggner, Rocio T. Martinez-Nunez, Simon R. Whiteoak, Jane E. Collins, JR Fraser Cummings, Tilman Sanchez-Elsner. MicroRNA control of the InterLeukin-13 pathway in Ulcerative Colitis. Awaiting Publication

7. Appendices

Appendix 1.

Version 5 (updated 1st Sept 2011) Date: __/__/__ Biopsy N°:_____

Biopsy Sample Questionnaire

1. Age _____
2. Sex ☐ male ☐ Female
3. Ethnicity ☐ white ☐ Asian ☐ black ____
4. Date of diagnosis _____
5. Previous surgery _____
6. Reason for scope _____
7. Diagnosis ☐ Crohn's ☐ UC ☐ Normal ☐ Not sure / other
8. Current drugs _____
9. Smoker ☐ yes ☐no ☐ex smoker[1]
10. Maximal extent of inflammation _____ Date _____
11. Normal day N° of stools _____ N° of loose stools _____
12. Last day N° of stools _____ N° of loose stools _____
13. Last week N° of stools _____ N° of loose stools _____
14. Bleeding[2] (in last day)
    N° of stools with blood _____
    ☐ no
    ☐ blood < ½ stools
    ☐ blood >1/2 stools
    ☐ blood only
15. On average how bad has your abdominal pain been over the last 7 days?
    ☐ None ☐ Mild ☐ Moderate ☐ Severe
16. On average how would you grade you well being over the past week?
    ☐ Well ☐ Slightly below par ☐ Poor ☐ Very poor ☐ Terrible
17. Other symptoms
    ☐ Arthritis or arthralgia
    ☐ Iritis or uveitis
    ☐ anal fissure, fistula or perirectal abscess
    ☐ other bowel-related fistula
    ☐ febrile (fever) episode over 38°C during past week
    ☐ erythema nodosum, pyoderma gangrenosum, aphthous stomatitis
    ☐ Nil
18. Taking Lomotil or opiates for diarrhea?
    ☐ Yes ☐ No
19. Abdominal mass?
    ☐ Yes ☐ No ☐ not sure
20. Physicians Global Assessment[3]
    ☐ normal (no symptoms of colitis, patient feels well, and scope score = 0)
    ☐mild disease (mild symptoms, scope score is mild)
    ☐moderate disease (more serious abnormalities and scope score and symptom scores of 1 or 2)
    ☐severe disease(symptom and scope scores of 3 and patient requires steroids and / or hospitalisation)
21. Blood tests taken __/__/__
22. Haematocrit _____
23. Weight change _____ (normal) _____ (actual)
24. Temperature _____
25. Pulse _____
26. Haemoglobin _____
27. ESR _____
28. CRP _____
29. Albumin _____
30. Creat _____ (80-115) or (53-97)
31. Other _____

Endoscopy

32. Findings
    _____
    _____
33. Max faegan score _____

Biopsy[4]

i. =
ii. =
iii. =

1 > 3 months ago
2 The most severe bleeding of the day

3 The PGA acknowledges 3 criteria, the patients daily record of abdominal discomfort and general sense of well being, and other observations, such as physicians findings and the patient's performance status.

4 0 = normal, 1 = granular mucosa, vascular pattern not visible, not friable, hyperaemic, 2 = AS 1 with friable mucosa but not bleeding, 3 = As 2 but mucosa spontaneously bleeding, 4 = As 3 with ulceration and denuded mucosa

1

Appendix 2.

National Research Ethics Service
Southampton & South West Hampshire REC (A)
Building L27
University of Reading
London Road
Reading
RG1 5AQ

Telephone: 0118 918 0567
Facsimile: 0118 918 0559

17 December 2010

Dr Fraser Cummings
Consultant Gastroenterologist
Southampton University NHS Trust
Gastroenterology Department
Mail Point 255
Southampton General Hospital
SO16 6YD

Dear Dr Cummings

Full title of study: **MicroRNA coordination of inflammation-mediated changes in human gut epithelial tight junctions and innate immune cytokine expression.**

REC reference number: **10/H0502/69**

Thank you for your letter of 9.11.2010. I can confirm the REC has received the documents listed below as evidence of compliance with the approval conditions detailed in our letter dated 14 September 2010. Please note these documents are for information only and have not been reviewed by the committee.

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Participant Information Sheet	2.0	21 July 2010

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

10/H0502/69	**Please quote this number on all correspondence**

Yours sincerely

William Goodyear
Committee Co-ordinator

E-mail: scsha.SWHRECA@nhs.net

Copy to: ***Ms Christine McGrath,***

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

Appendix 3.

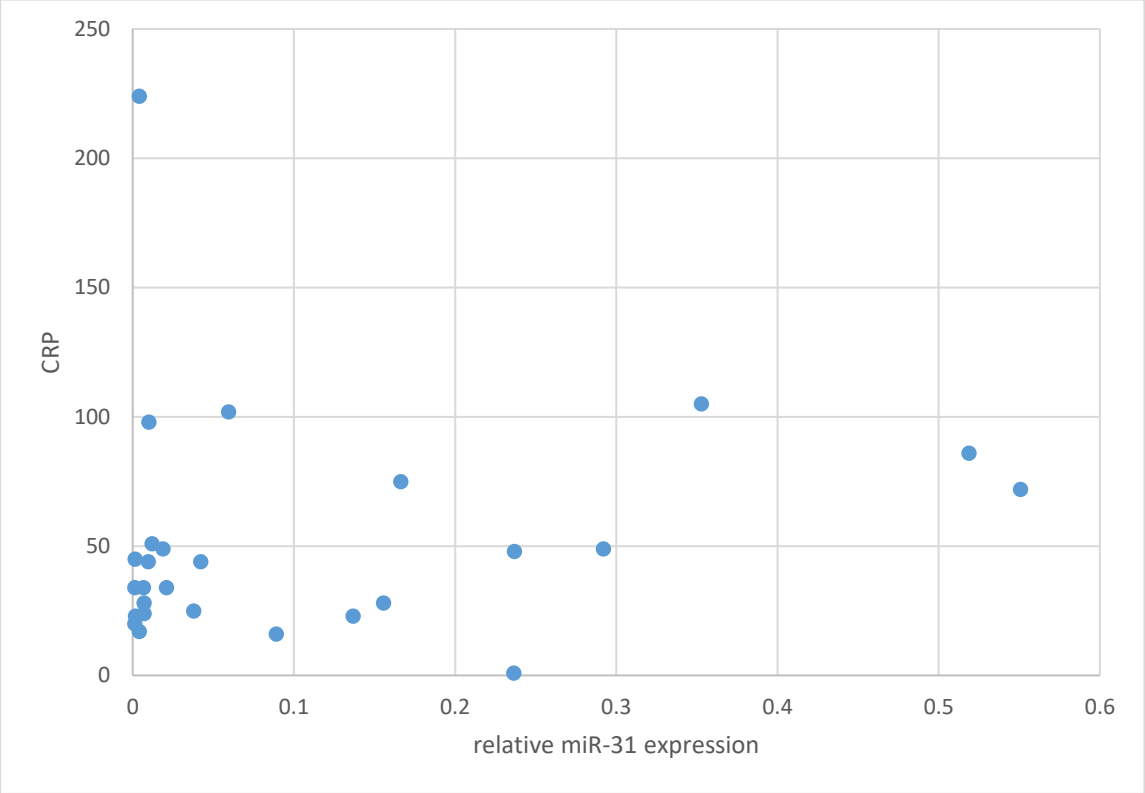


Figure 90. Regression curve comparing CRP and relative miR-31 expression in patient with Crohn's Colitis

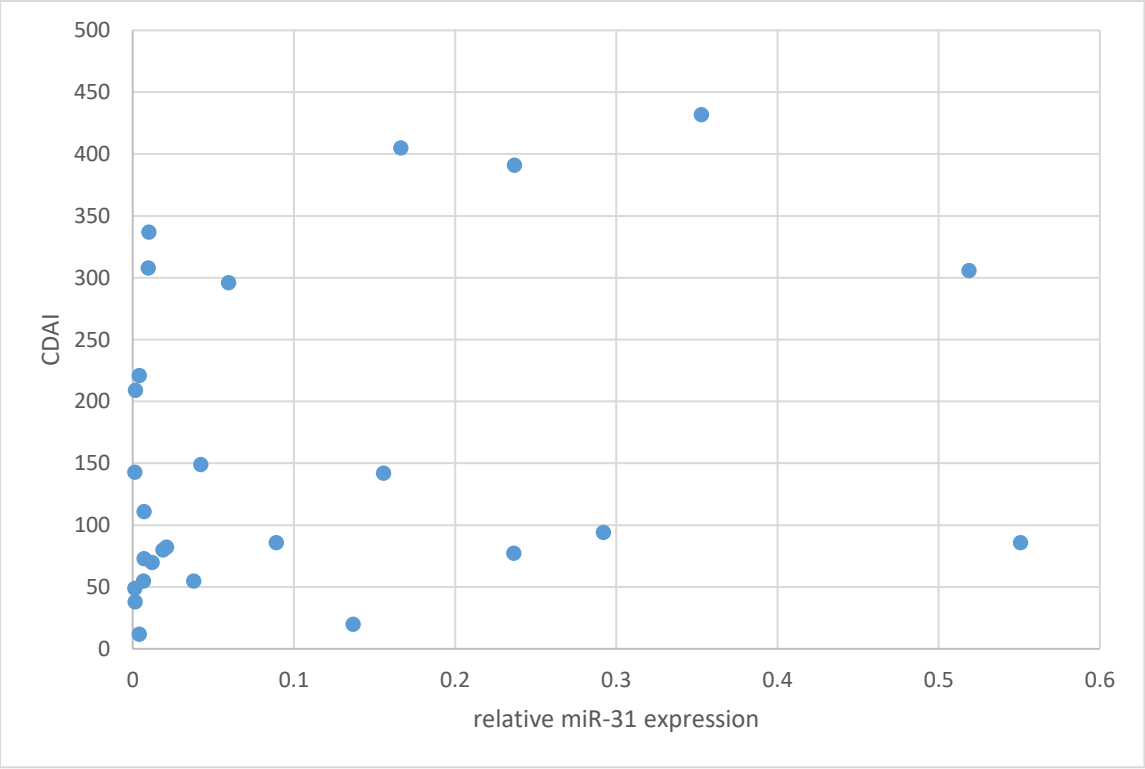


Figure 91. Regression curve comparing CDAI and relative miR-31 expression in patient with Crohn's Colitis

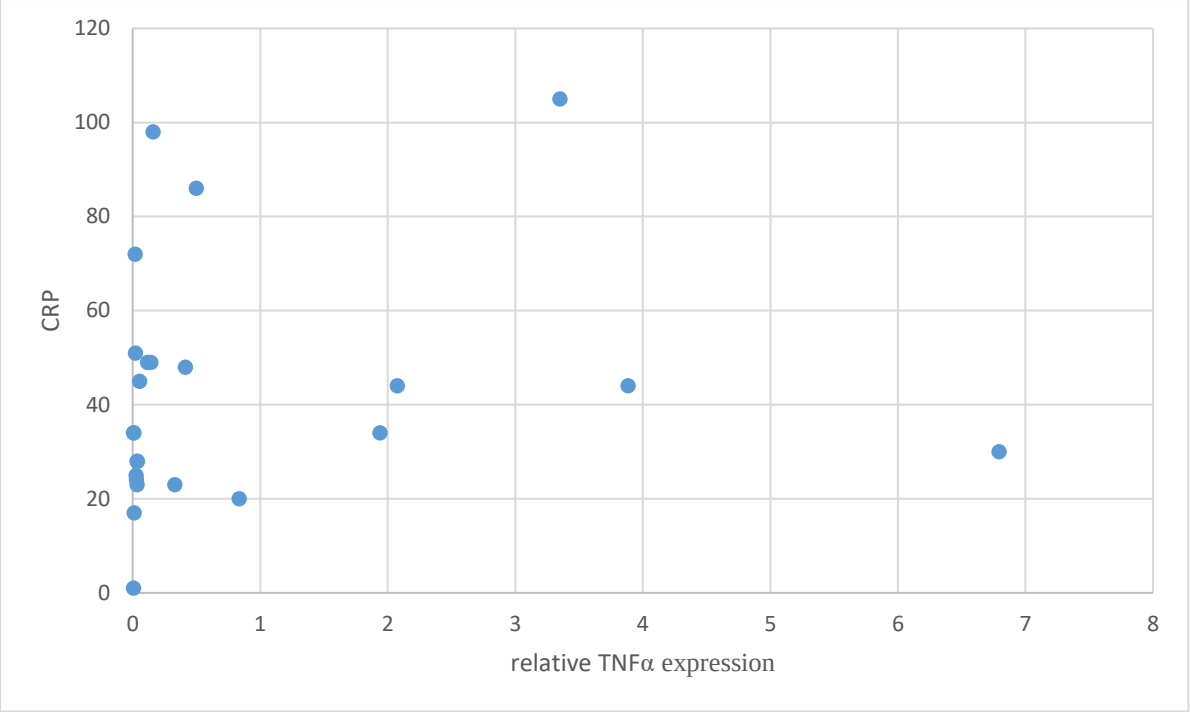


Figure 92. Regression curve comparing CRP and relative TNFα expression in patient with Crohn's Colitis

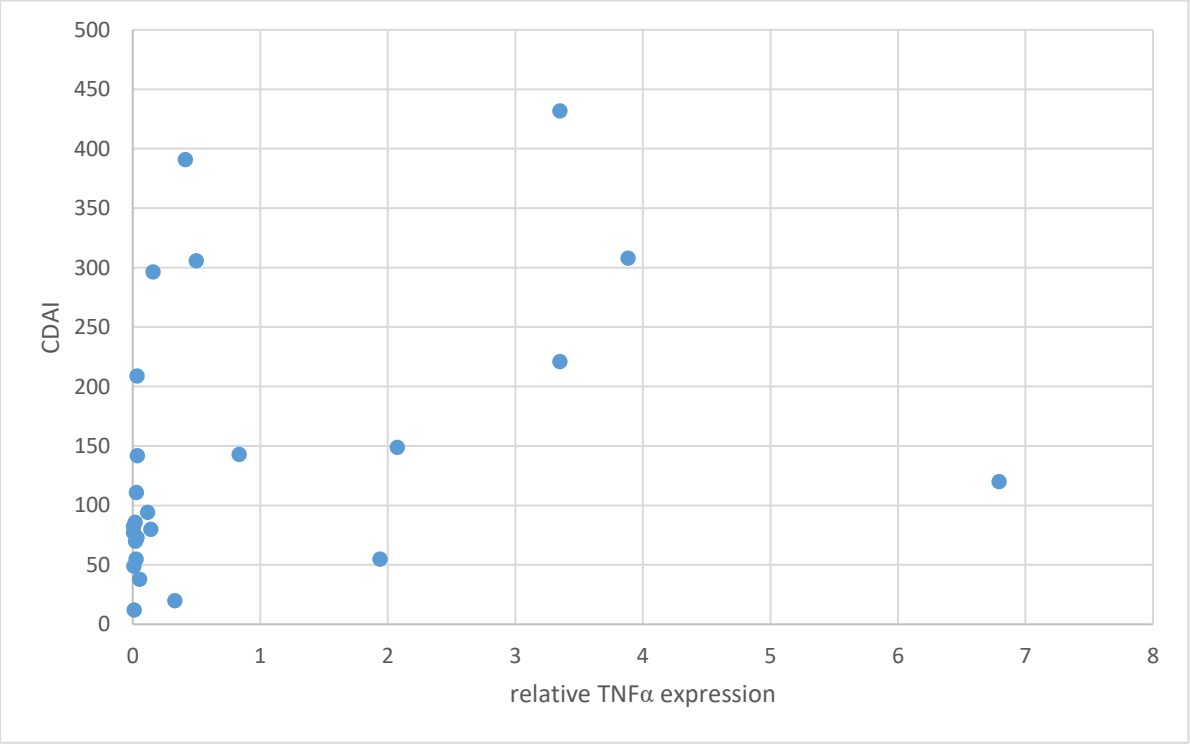


Figure 93. Regression curve comparing CDAI and relative TNFα expression in patient with Crohn's Colitis

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