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**UNIVERSITY OF SOUTHAMPTON**

**FACULTY OF MEDICINE**

**School of Medicine**

**A study of the role of microRNAs in  
Inflammatory Bowel Disease: The effect  
of miR-31 dysregulation in the  
expression of TSLP in Ulcerative Colitis**

**By**

**Dr Andrew Claridge**

**PhD thesis**

**June, 2013**



**Dedicated to Sherry for her support, Abigail for her cuddles and Tristan for his smiles every morning.**



UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

Clinical and Experimental Sciences

Doctor of Philosophy

A STUDY OF THE ROLE OF MICRORNAS IN INFLAMMATORY BOWEL DISEASE: THE EFFECT OF MIR-31 DYSREGULATION IN THE EXPRESSION OF TSLP IN ULCERATIVE COLITIS

By Andrew Claridge

Our current understanding suggests that Inflammatory Bowel Disease (IBD) is an immunological mediated disease triggered by unknown environmental factors in those who are genetically susceptible. MicroRNAs (miRNAs) are a class of small non coding RNAs that play a critical role in many immunological pathways by regulating gene expression. To date over 70 miRNAs have been identified as having a differential expression in IBD although their roles remain unknown.

Using microarray and RT-qPCR we have identified the differential expression of 7 miRNAs; miR-31, -146b, -194, -200b, -223, -375 and -422a, in the sigmoid mucosa of treatment naïve Ulcerative Colitis. Of these, the expressions of miR-31 and -223 are also increased in mucosal lymphocytes and the CD4<sup>+</sup>CD25<sup>intermediate</sup> subfamily.

Using *in silico* analysis, thymic stromal lymphopoietin (TSLP) is identified as a target for 4 of the 7 miRNAs; miR-31, -223, -194 and -422a. TSLP has been shown to be increased in T<sub>H</sub>2 mediated disorders of the lung and skin and implicated in epithelial barrier homeostasis and lymphocyte function in response to luminal flora. Here we show for the first time the expression of TSLP by healthy lymphocytes and a decreased expression in mucosal biopsies and mucosal lymphocytes in active UC. In addition, using a human lymphocyte cell model we demonstrate an increased expression of miR-31 in stimulated lymphocytes which is inversely related to a decreased expression of TSLP mRNA and an even larger

## **Abstract**

effect on the expression of TSLP protein in the same cells. Selective inhibition of miR-31 prevents the decrease in TSLP in stimulated lymphocytes.

The increased expression of miR-31 in UC actively decreases the expression of TSLP, a factor associated with epithelial homeostasis and less destructive inflammation. Manipulation of the miR-31 / TSLP pathway offers a potential therapeutic strategy for UC.

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# Declaration of Authorship

I, Andrew Claridge declare that the transfer thesis entitled “The role of miRNA in Inflammatory Bowel Disease”, and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University;
- No part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given;
- With the exception of such quotations, this thesis is entirely my own work unless otherwise stated;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

Signed:

Date: 24<sup>th</sup> June 2013

# Acknowledgments

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I'm often asked what I have gained from taking time out of clinical practice to complete a PhD, the superficial answer is, a large overdraft, 2 children and a cat. However, the most important lesson a period of research has taught me is to look beyond the obvious and behind what you are being presented, often the most important information is hidden in the detail. Good clinicians will always say the secret to a correct diagnosis is a good history and examination, likewise in science, you can only draw reliable results from good experiments. The parallel between IBD at the bedside and experiments in the lab is a good example of this. At the bedside the relevance of an investigation depends on the clinical context, and similarly in the lab the conclusion drawn from an experimental result depends on the methodology and assumptions.

So as well as the overdraft, the children and the cat, I have also gained a great deal of knowledge, an appreciation of scientific methodology, an understanding of how to interpret conclusions and most importantly a much better grasp of how science applies to the real world.

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AC

# Abbreviations

AIM2	Absent in melanoma 2
Alb	Albumin (g/l)
APC	Antigen presenting cell
ATG16L1	Autophagy-related protein 16-1
BCR	B cell receptor
$\beta$ -ACT	Beta actin
BIR	Baclovirus inhibitor of apoptosis protein repeat domain
BMI	Body mass index
BSG	British Society of Gastroenterology
cAMP	Cyclic adenosine monophosphate
CARD	Caspase recruitment domain
CD	Crohn's disease
CD#	Cluster of differentiation <i>followed by a number</i>
CDAI	Crohn's disease Activity Index
CDEIS	Crohn's disease endoscopic index of severity
cDNA	Complimentary DNA
CIITA	Class 2, major histocompatibility complex, transactivator
Cmf	Calcium and Magnesium free
Cr	Creatinine
CRP	C reactive protein (mg/hr)
Ct	Threshold cycle
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4 (CD152)
CXCL#	Chemokine (CXC motif) ligand <i>followed by a number</i>
DAI	Disease Activity Index (Mayo score)
DPBS	Dulbecco's Phosphate-Buffered Saline
DGCR8	DiGeorge Syndrome Critical Region 8
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Dextran Sulphate Sodium
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate (mm/hr)
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine (nee Calf) serum
FMRP	fragile X mental retardation protein
FITC	Fluorescein isothiocyanate

## Abbreviations

FoxP3	Forkhead box p3 protein encoded by the gene FOXP3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA3	Transacting T cell specific transcription factor GATA3
G-CSF	Granulocyte colony stimulating factor
GI tract	Gastrointestinal tract
GITC	Guanidine isothiocyanate
GITR	Glucocorticoid induced TNF receptor
GM-CSF	Granulocyte macrophage colony stimulating factor
GOI	Gene of interest
Hb	Haemoglobin (g/l)
HBI	Harvey Bradshaw Index
HBSS	Hank's Balanced Salt Solution
Hct	Haematocrit
HLA-###	<i>Followed by a number</i>
HO-1	Heme oxygenase -1
IBD	Inflammatory Bowel Disease
ICOSLG	ICOS (inducible T-cell costimulator) ligand (CD275)
IEL	Intra-epithelial lymphocytes
IL-#	Interleukin- <i>followed by a number</i>
INF- $\gamma$	Interferon gamma
IPAF	Ice protease activating factor, also known as NLRC4 (NLR family CARD domain containing protein 4)
IPEX	immunodysregulation polyendocrinopathy and enteropathy X-linked syndrome
IQR	Inter quartile range
IRAK	IL-1 associated kinase
IRF#	Interferon regulatory factor <i>followed by a number</i>
IRGM	Immunity related GTPase family M protein
JAK2	Janus Kinase 2
JNK	C Jun N terminal kinases
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MDP	Muramyl dipeptide
Mecp2	Methyl CpG binding protein 2
MGB	Minor groove binder
MHC	Major histocompatibility complex
MIP-2	Macrophage inhibitory protein
MIP-2	Macrophage inhibitory protein -2
miR-###	mature micro RNA <i>followed by a number</i> , usually the number indicates the chronological order of discovery

miRNA	microRNA
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MW	Mann Whitney U test
MyD88	Myeloid differentiation primary response gene (88)
NACHT	Domain named after NAIP, C2TA, HET-E and TP1, alternative name for NOD domain
NAIP	Neuronal apoptosis inhibitory protein
NALP	A NLR
NEMO	NF- $\kappa$ B essential modulator
NF- $\kappa$ B	Nuclear factor $\kappa$ B
NK	Natural killer
NKX2.3	Homeobox protein nkx-2.3
NLR	NOD like receptor
NOD	Nucleotide binding oligomerization domain
ORMDL3	ORM1 like protein 3
PACT	protein activator of the interferon induced protein kinase
PAMP	Pathogen associated molecular pattern
PE	R-phycoerythrin
PB	Pacific Blue
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered solution
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
PRDM1	PR domain zinc finger protein 1
PRR	Pattern recognition receptor
PSC	Primary Sclerosing Cholangitis
Psmb9	Proteasome subunit beta type 9
PYD	Pyrin domain
REL	Proto-oncogene c-Rel
RIG-1	Retinoic acid inducible gene 1
RIP2	Receptor interacting protein 2
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROR#	Retinoic acid receptor related orphan receptors <i>followed by a number</i>
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT-qPCR	Real Time - quantitative PCR

## Abbreviations

sd	Standard deviation
SES-CD	Simple endoscopic score for Crohn's disease
SMAD#	Mother against decapentaplegic homolog 1 <i>followed by a number</i>
SOCS1	Suppressor of cytokine signalling 1
spp.	Species
STAT#	<i>Followed by a number</i>
TCR	T cell receptor
TGF- $\beta$	Transforming growth factor beta
T <sub>H</sub> #	Helper T lymphocyte <i>followed by a number</i>
TICAM-#	<i>Followed by a number</i>
TIR	Toll / interleukin 1 receptor
TLR	Toll like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF- $\alpha$	Tumour necrosis factor alpha
TRAF6	TNF associated factor protein 6
TRBP	transactivating response RNA binding protein
T <sub>reg</sub>	Regulatory T lymphocyte
TRIF	TIR domain containing adapter inducing interferon
Tudor-SN	Tudor staphylococcal nuclease-domain-containing protein
TYK2	Tyrosine kinase 2
U&Es	Renal function and serum electrolytes
UC	Ulcerative Colitis

# Terminology

For clarity, certain words used in this document have the following definitions

Antigen	A substance which stimulates the production of an antibody.
Pathogen	A microorganism that causes disease.
Inflammatory Bowel Disease / IBD	A collective term for UC and Crohn's disease, which does not include any other cause of inflammation of the intestine.
Active UC / Crohn's / IBD	Macroscopic inflammation at the site of the biopsy.
Inactive UC / Crohn's / IBD	No macroscopic inflammation within any part of the intestine seen during the endoscopy, but the patient has an established diagnosis of either UC or Crohn's.
Unaffected UC / Crohn's / IBD	The biopsy is taken from a site where there is no macroscopic inflammation, but there is inflammation in other parts of the intestine.
RT-PCR	Reverse Transcription - PCR. Not to be confused with real time - PCR which is also often abbreviated to RT-PCR in the literature.
miRNA seed region	The consecutive 7-8 nucleotide sequence starting at the 1 <sup>st</sup> or 2 <sup>nd</sup> base of the 5' end of a miRNA.
Candidate miRNAs	The 7 miRNAs identified by microarray to be differentially expressed in active sigmoid UC compared with healthy controls. Candidate miRNA are miR-31, miR-146b, miR-194, miR-200b, miR-223, miR-375, miR-422a.
Colitis	Colitis is an umbrella term which means "inflammation of the colon". Colitis has many distinct forms and manifestations depending on the aetiological factors, i.e. infection, toxin, microscopic colitis and radiation colitis. IBD is a distinct category of colitis which includes, in this thesis, only UC and Crohn's disease. In the wider literature, IBD and colitis are often used interchangeably, especially in the literature that review or report IBD. In this thesis the term "colitis" is used in reference to non UC and non Crohn's disease forms of inflammation of the colon.
Relative expression / fold change	Differential expression ( $2^{-\Delta\Delta CT}$ ) of mRNA or miRNA after normalising to a house keeper gene and comparing against control.
Treatment naive	No medical treatment for IBD for $\geq 6$ months



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## **Chapter 1. Introduction**

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## 1.1 Inflammatory Bowel Disease

### 1.1.1 Introduction

Crohn's disease (Crohn's) and Ulcerative Colitis (UC) are the two major types of chronic idiopathic Inflammatory Bowel Disease (IBD). Together UC and Crohn's affect approximately 1 in 250 people in the UK, and have a wide ranging and often devastating impact on health and quality of life (Ghosh and Mitchell, 2007, Claridge, 2010). It is a disease of the young and for many inflicts a lifetime of relapsing and remitting activity (Solberg et al., 2007).

Rare before the 1940's the incidence of IBD rose steadily until the 1980's, for UC it then reached a plateau, but for Crohn's the incidence has continued to rise slowly. In the UK the current prevalence for UC is 243 per 100,000 and for Crohn's 145 per 100,000 (Rubin et al., 2000). The financial burden of IBD is increasing and likely to grow exponentially with the adoption of more sophisticated investigative techniques and modern management regimes. Current estimates place the cost to the NHS of between 500 and 700 million pounds per year, although the added cost to the individual, including indirect and non-medical costs may be as much as 2 to 3 times this amount (Bassi et al., 2004, Stark R. et al., 2006).

Predominantly a disease of northern Europeans, there are an estimated 2.2 million people with IBD across Europe (Loftus, 2004). The global impact of IBD is less well known and is still considered to be a rare illness in Africa, South America and the Far East; however, over recent decades the incidence in these areas is increasing, trebling in the last 10 years. Although the prevalence elsewhere is still well below that of northern Europe, IBD is now a recognised and growing problem worldwide (Ahuja and Tandon, 2010).

Historical data suggests that in the untreated patient the short term mortality from an acute attack of IBD is similar to that of a Myocardial Infarction, and the longer term mortality is much greater. Acute myocardial infarction treated with placebo has a 35 day mortality of 11.8% (ISIS-2, 1988) and a 1 year mortality of 18.6% (Baigent et al., 1998), compared with 9.9% at 42 days and 23.7% at 9 months for severe UC. In addition to the 1 in 4 people who have died as a result of their

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initial attack of UC another half remain symptomatic after 9 months (Truelove and Witts, 1955).

In the early 1950s a steroid, cortisone, was introduced for the treatment of UC and reduced the mortality rate from 23.7% to 7% and increased symptom free remission to almost 50% at 9 months (Truelove and Witts, 1955). The treatment of Crohn's with steroids followed shortly afterwards, although it took another 20 years for a randomised controlled trial to demonstrate a definitive benefit (Summers et al., 1979).

Over the years many medical and non-medical treatments have been introduced for IBD, however the success has been variable and many difficulties remain. Traditional treatments are unsatisfactory; often any potential benefits are offset by unacceptable side effects, and newer biological treatments, such as anti-TNF therapies, are only effective in up to 60% of patients in the short term (Rutgeerts et al., 2005), and over time loose efficacy as patients develop tolerance or side effects (Zabana et al., 2010).

Given the impact of IBD on patients, their families and society as a whole, there is considerable interest to understand the aetiology of IBD with a view to developing new therapies.

### 1.1.2 Classification, definition and diagnosis

The term "Inflammatory Bowel Disease", is an accepted collective term for UC and Crohn's, and used throughout this thesis with this definition. The term is often preceded by the word idiopathic to distinguish the diseases from other forms of inflammatory bowel disease such as those caused by infection, toxin or ischemia. All of which can often be indistinguishable from IBD.

Idiopathic ulceration of the colon later to be coined Ulcerative Colitis was first described in 1859 by Wilks and Moxon (cited by Asakura et al., 2007). Isolated inflammation of the terminal ileum as a separate entity and not caused by TB, was first described in 1913 by Dalziel (Dalziel, 1913), although in 1932 this pattern of disease was given the name Crohn's after Burrill Crohn, Ginzburg, and Oppenheimer gained notoriety for describing a case series of idiopathic regional

ileitis associated with intestinal stenosis and fistulae (Crohn et al., 1932). Despite these relatively recent histories, case reports of illnesses, likely to be IBD, go back many centuries<sup>1</sup>, and illnesses with striking similarity to IBD are described back to antiquity.

The classical difference between UC and Crohn's is the disease distribution, although microscopic appearance, macroscopic appearance, the depth of inflammation seen by imaging or surgery, strictures, fistulae and extra-luminal features are often used in combination to help distinguish between the two. However, there are no findings which can definitively differentiate UC from Crohn's, and the significant overlap in clinical phenotype, investigative findings, and disease progression result in 10-15% of patients having their disease label changed within a year of diagnosis and approximately 5% of patients being labelled as "IBD, type unclassified" in the longer term.

To complicate things further the differences and overlap of the clinical phenotype and investigative findings of both UC and Crohn's are dependent on the population in question. For example the age of onset or disease distribution in Asian compared with Western populations is reviewed by Ahuja and Tandon (2010), the disease distribution can be altered by treatment; e.g. the skip lesions or rectal sparing classically associated with Crohn's are often found in partially or locally treated UC respectively. There are also disease variants in which the classical features of one disease are a feature of the other, for example, terminal ileitis is seen in up to 1 in 5 patients who have undergone resection for extensive UC but can also be found in association with distal disease only (Haskell et al., 2005).

Other difficulties of interpretation are the differing classification criteria between observers and different causative and propagating factors between population groups. All of the above complicate the interpretation of studies which rarely define their population groups in terms of race, disease definition or disease phenotype.

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<sup>1</sup> The first description of CD is attributed to an Italian physician Giovanni Battista Morgagni in 1769, when he investigated and diagnosed a man with a chronic, debilitating illness and diarrhoea. (Article Source: <http://EzineArticles.com/409008>, accessed 2.9.11)

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Despite the diagnostic problems our increased understanding of the disease process has enabled us to increasingly use new biological characteristics including; biochemistry, immunology and genetic variations to differentiate patients into UC or Crohn's. Differentiating disease and defining phenotype is critical in planning and predicting the response to treatment, and the challenge reflects the complex interplay between aetiological and propagating factors.

### 1.1.3 Ulcerative Colitis

#### 1.1.3.1 Introduction

The age at which Ulcerative Colitis presents has a bimodal distribution; the peak age of presentation is between 15 and 25 years of age with a second peak between 50 and 60 years of age. The classical presentation includes diarrhoea often with visible blood, perhaps some weight loss and occasional abdominal pain. Ulcerative Colitis is characterised by diffuse mucosal inflammation of the colon, without involving the ileum. The inflammation of treatment naïve UC is not patchy unlike the pattern in Crohn's. The three most clinically relevant characteristics of UC are; disease extent (which in the case of UC correlates to phenotype), clinical symptoms and endoscopic appearance.

#### 1.1.3.2 Phenotype

Prognosis and treatment outcomes are closely related to phenotype which is generally accepted as being characterised by the disease extent, i.e. the length of colonic mucosa affected, beginning from the rectum, which is diffusely inflamed. This is the most easily measured variable and most commonly used clinically.

Maximal extent of inflammation observed at colonoscopy	
Proctitis	E1
Left-sided - extending to splenic flexure	E2
More extensive disease	E3

Table 1. Definition of UC according to the Montreal classification (Silverberg et al., 2005).

Disease extent is classified using the Montreal classification, Table 1 (Silverberg et al., 2005). Disease extent has significant implications for a variety of variables including lifetime risk of developing Colorectal cancer, optimising the use of topical treatments, response to treatment, risk of requiring colectomy and long term morbidity (Farmer et al., 1993, Biancone et al., 2008).

There are some less common variants of UC, for example those patients who also suffer from UC associated Primary Sclerosing Cholangitis (PSC), and / or other extra intestinal manifestations in which IBD may be part of a syndrome rather than a distinct disease in itself (Evans and Pardi, 2007). These phenotypic variants are most likely to represent different disease aetiologies and are therefore not included or considered further.

### 1.1.3.3 Clinical activity in UC

There is no internationally agreed system of defining clinical disease activity in UC, the method used is often dependent on the context in which activity is being calculated (Travis et al., 2011). Of the numerous activity indexes which are used, both the Food and Drug Administration (USA) and the British Society of Gastroenterology (UK) currently recommend disease activity assessment using either the Truelove and Witts clinical activity index, Table 2, or the Mayo score disease activity index known as DAI, Table 3.

Activity	Criteria
Severe	<p>≥6 motions a day with macroscopic blood in stools.</p> <p>And ≥ 1 of the following;</p> <p>Fever (mean evening temperature &gt;37.5 °C), or a temperature of ≥37.8 °C on ≥ 2 days out of 4.</p> <p>Tachycardia (pulse &gt;90bpm)</p> <p>Anaemia (Hb ≤75% normal)</p> <p>E.S.R. ≥ 30mm/hr</p>
Moderately severe	Intermediate between severe and mild.
mild	≤4 motions a day with no more than small amounts of macroscopic blood in stools. No fever. No tachycardia. Anaemia not severe. E.S.R. ≤30

Table 2. The Truelove and Witts clinical activity index for UC (Truelove and Witts, 1955).

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The Mayo score disease activity index (DAI)	
Stool frequency	0= normal no. of stools for this patient 1= 1-2 stools more than usual 2= 3-4 stools more than usual 3= 5 or more stools than normal
Rectal bleeding <sup>1</sup>	0= no blood seen 1= streaks of blood with stool less than half the time 2= obvious blood with stool most of the time 3= blood alone passed
Scope findings	0= normal or inactive disease 1= mild disease (erythema, decreased vascular pattern, mild friability) 2= moderate disease (marked erythema, absent vascular pattern, friability, erosions) 3= severe disease (spontaneous bleeding, ulceration)
Physicians global assessment <sup>2</sup>	0= normal (no symptoms of colitis, patient feels well, and scope score = 0) 1= mild disease (mild symptoms, scope score is mild) 2= moderate disease (more serious abnormalities and scope score and symptom scores of 1 or 2) 3= severe disease (symptom and scope scores of 3 and patient requires steroids and / or hospitalisation)

<sup>1</sup>The most severe bleeding of the day

<sup>2</sup>The PGA acknowledges 3 criteria, the patient's daily record of abdominal discomfort and general sense of well being, and other observations, such as physician's findings and the patient's performance status.

Table 3. Mayo score disease activity index (DAI) for UC (Schroeder et al., 1987).

Cortisone was the new wonder drug for many inflammatory conditions just after the 2nd World War<sup>2</sup>. The first randomised controlled trial of cortisone in IBD was published by Truelove and Witts (1955) who also introduced the first disease activity index the "Truelove and Witts clinical activity index", which is still widely used today, The index was developed to identify those patients with high acute mortality and those requiring colectomy. Its ability to identify severe disease is well validated; however it is limited in its ability to discriminate mild or moderate disease or changes in activity over time and therefore many other indices have been used, or proposed, which incorporate numerous other prognostic markers (Caprilli et al., 2007, Cooney et al., 2007). Despite its short comings the Truelove and Witts activity index is still the most widely utilised clinical activity index in the setting of severe UC.

<sup>2</sup>The advent of modern medicine were exemplified in "Bigger Than Life" (1956), a movie classic starring James Mason and Walter Matthau that tells the story of a small town school teacher whose life is saved from the rapidly fatal effects of polyarteritis nodosa, by the prescription of an "experimental" new drug, cortisone..

## 1.1.3.4 Endoscopic activity index

Endoscopic activity is now gaining favour as the most important factor in the prediction of disease activity and treatment outcome, and is becoming a more commonly used activity marker in clinical practice. Endoscopic activity is also widely used as a disease activity marker and endpoint in many important trials.

	Baron Score (Baron et al., 1964)	Modified Baron / Feagan Score (Feagan et al., 2005)	Mayo score (Schroeder et al., 1987)
0	Normal matt mucosa, ramifying vascular pattern clearly visible, no spontaneous bleeding, no bleeding to light touch	Normal smooth glistening mucosa with vascular pattern visible, not friable	normal or inactive disease
1	Abnormal, but non-haemorrhagic appearance between 0 and 2	Granular mucosa, vascular pattern not visible, not friable, hyperaemia	mild disease (erythema, decreased vascular pattern, mild friability)
2	Moderately haemorrhagic, bleeding to light touch, but no spontaneous bleeding seen ahead of the instrument on initial inspection	As 1, with a friable mucosa, but not spontaneously bleeding	moderate disease (marked erythema, absent vascular pattern, friability, erosions)
3	Severely haemorrhagic, spontaneous bleeding seen ahead of instrument at initial inspection and bleed to light touch	As 2, but mucosa spontaneously bleeding	severe disease (spontaneous bleeding, ulceration)
4		As 3, but clear ulceration, denuded mucosa	

Table 4. A comparison of the 3 most commonly used endoscopic scoring algorithms.

There are over 9 widely used scoring systems for endoscopic activity in UC, the most widely used are the Baron scoring system introduced by Baron et al. (1964) from which many adaptations have been developed including the modified Baron score, otherwise known as the Feagan score (Feagan et al., 2005), and the Mayo score taken from the endoscopic section of the Mayo activity index, Table 4.

Although endoscopic activity is rapidly gaining favour over clinical activity indexes as the desired end point for both clinical treatment as well as trials there are a number of recognised drawbacks. The first is the substantial inter-observer variation in the endoscopic assessment of severity. Bernhardt et al (2009) reported

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that for severe colitis there is only 76% concordance between endoscopists, and only 47% concordance for moderate inflammation. The second is the difficulty in comparing different indexes, clinical indexes tend to give trials a more favourable outcome and have therefore been favoured historically in pharmaceutical studies. Clinical indexes cannot be compared with endoscopic indexes because symptoms correlate poorly with endoscopic (and biochemical) activity.

### 1.1.4 Crohn's Disease

#### 1.1.4.1 Introduction

Crohn's disease most commonly presents in early adulthood and is characterised by a transmural and granulomatous patchy inflammation of any part of the gastrointestinal tract (GI tract). Crohn's disease phenotype can be more clearly subdivided than UC, mainly due to the clear distinctions between different anatomical distributions and the complications that ensue. In Crohn's approximately 30% of patients present with endoscopic and / or radiological evidence for ileal disease, 45% with involvement of both the ileum and colon, largely localised to the terminal ileum and IC valve, and 26% present with isolated colonic inflammation (Truelove and Pena, 1976).

The most common presenting symptom is diarrhoea, seldom associated with blood. Abdominal pain and weight loss are also commonly seen, and are more frequent complaints than in UC. Extra intestinal manifestations are also more common place mainly involving the joints, skin and eyes.

The cardinal difference between UC and Crohn's is the depth of inflammatory involvement. Due to transmural inflammation seen in Crohn's, penetrating (fistulae) and strictures are frequently encountered, and when present, drastically alter the disease impact, treatment and disease course.

#### 1.1.4.2 Phenotype

An initial consensus meeting in Rome 1991 identified; age of onset, disease location and behaviour as the three main phenotypic characteristics that affected disease prognosis and treatment outcome in Crohn's. Reviewed in Vienna 1998

(Gasche et al., 2000) and updated in Montreal 2005 (Silverberg et al., 2005), the simplified Montreal classification is now used to define phenotype in Crohn's, Table 5.

Age of onset		location		Behaviour	
<16 years	(A1)	Ileal	(L1)	Non-stricturing	
17-40 years	(A2)	Colonic	(L2)	Non penetrating	(B1)
>40 years	(A3)	Ileocolonic	(L3)	Stricturing	(B2)
				Penetrating	(B3)
				Perianal disease	+ p

Table 5. Definition of Crohn's disease phenotype according to the Montreal classification (Silverberg et al., 2005).

Like UC the distribution of phenotypes differs between population groups, and again may reflect different aetiological and propagating factors.

#### 1.1.4.3 Clinical activity in Crohn's disease

In clinical practice Crohn's activity is graded as mild, moderate or severe. The 2006 European Crohn's and Colitis Organisation guidance definitions are outlined in Table 6, and although helpful in clinical situations are subjective and are subject to large inter-observer variation.

Mild	Moderate	Severe
Equivalent to a CDAI of 150–220	Equivalent to a CDAI of 220–450	Equivalent to a CDAI of >450
Ambulatory, eating and drinking.	Intermittent vomiting, or weight loss >10%. Treatment for mild disease ineffective, or tender mass.	Cachexia (BMI <18)
<10% weight loss.	No overt obstruction.	Evidence of obstruction or abscess. Persistent symptoms despite intensive treatment.
No features of obstruction, fever, dehydration, abdominal mass, or tenderness.	CRP raised above the upper limit of normal.	Raised CRP.
CRP usually increased above the upper limit of normal		

Table 6. Clinical grading of Crohn's disease (Adapted from Strange et al. (2006) for CDAI score see Table 7.

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In clinical studies or in patients who are being assessed or monitored for biological therapy Crohn's activity is measured more objectively using the Crohn's Disease Activity Index (CDAI), Table 7, or Harvey Bradshaw Index (HBI), Table 8.

Almost 4 decades ago Best et al. (1976) developed the Crohn's activity index (CDAI), they monitored 18 variables in approximately 120 patients who they consulted for both in- and out-patient treatment. Eight variables were incorporated into a rather complex equation to achieve an activity score. Despite the possibility that symptoms not due to active disease can cause an increase in the CDAI, it remains the principle index used for trials in Crohn's, although more recently the CRP has been incorporated into the index to reduce the false positive response seen by placebo. It is now accepted that CDAI >150 is required for a definition of active disease and a change >100 points is required to indicate a response to treatment, although some studies use different values which complicate comparison.

Clinical or laboratory variable	Weighting factor
Number of liquid or soft stools each day for seven days	x 2
Abdominal pain (graded from 0-3 on severity) each day for seven days	x 5
General well being, subjectively assessed from 0 (well) to 4 (terrible) each day for seven days	x 7
Presence of complications*	x 20
Taking Lomitil or opiates for diarrhoea	x 30
Presence of an abdominal mass (0 as none, 2 as questionable, 5 as definite)	x 10
Absolute deviation of haematocrit from 47% in men and 42% in women	x 6
Percentage deviation from standard weight	x 1

\*One point each is added for each set of complications;

- the presence of 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 (> 100 °F) during the previous week.

Table 7. The Crohn's Disease Activity Index (CDAI).Adapted from Best et al. (1976).

The Harvey Bradshaw Index is a five variable scoring index which is easy to use and applicable to the bed side. The first three items are scored for the previous day. Remission, mild disease, moderate disease and severe disease are accepted as a HBI score of <5, 5-7, 8-16 and >16 respectively.

variable	scores
General well-being	0 = very well, 1 = slightly below par, 2 = poor, 3 = very poor, 4 = terrible
Abdominal pain	0 = none, 1 = mild, 2 = moderate, 3 = severe
Number of liquid stools per day.	1x number
Abdominal mass	0 = none, 1 = dubious, 2 = definite, 3 = definite and tender
Complications: arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure, new fistula, abscess	score 1 per item

Table 8. The Harvey Bradshaw Index (HBI).

#### 1.1.4.4 Endoscopic activity index

Endoscopic activity is more difficult to define for Crohn's than UC because inflammation may be patchy, involve areas of the GI tract beyond the reach of conventional endoscopy and may have overlying normal mucosa in the presence of submucosal disease. Despite the diagnostic difficulties, mucosal lesions are currently the standard way of measuring disease activity and healing (Daperno et al., 2011), and correlate strongly with disease prognosis (Ardizzone et al., 2004, Peyrin-Biroulet et al., 2011). The Crohn's disease endoscopic index of severity (CDEIS) (Mary and Modigliani, 1989), or the simple endoscopic score for Crohn's disease (SES-CD) (Daperno et al., 2004), Table 9, are the most widely used in clinical trials. Although in clinical practice, endoscopic activity is graded by the presence and appearance of ulcers and / or inflammatory activity in each region using the Mayo score, Table 3.

## Chapter 1. Introduction

	0	1	2	3
Size of ulcers	None	Aphthous ulcers (Ø 0.1 -0.5cm)	Large ulcers (Ø 0.5 - 2.0cm)	Very large ulcers (Ø >2cm)
Ulcerated surface	None	<10%	10-30%	>30%
Affected surface	Unaffected segment	<50%	50-75%	>75%
Presence of narrowing	of none	Single and can be passed	Multiple, can be passed	Cannot be passed

5 predefined endoscopic segments, the Ileum, right colon (IC valve, caecum and ascending), transverse, left colons and the rectum. Each section was given a score of 0-3 from the table above.

Table 9. The simple endoscopic score for Crohn's disease (SES-CD) (Daperno et al., 2004).

### 1.1.5 The histopathology of IBD

Histological examination in IBD serves a number of functions; supporting the diagnosis, differentiating between UC and Crohn's, assessing disease activity and excluding alternative diagnoses or the presence of dysplasia.

Histology is not a prerequisite for diagnosis and by itself only achieves a diagnostic sensitivity of up to 74% for UC and between 18% and 62% for Crohn's (Bentley et al., 2002). Differentiation between UC and Crohn's by histology alone is also difficult due to the lack of defining histological characteristics. The presence of an epithelioid granuloma is specific to Crohn's although only a feature in 18% of patients and is easily confused with peri-cryptic histiocytic granuloma or histiocytic reaction around ruptured crypts, both commonly seen in UC and over 50% of Crohn's depending on disease severity and the source of the tissue (Molnar et al., 2005). Besides epithelioid granuloma, patchy inflammation is another commonly used feature to differentiate UC from Crohn's although this again is common to both diseases depending on biopsy site and treatment regime.

In circumstances where inflammatory activity is measured, there are four criteria which are assessed; architectural abnormalities, signs of chronic inflammation, signs of acute inflammation and epithelial abnormalities, these are outlined in more detail by Jenkins et al. (1997). These 4 histological criteria are combined in a number of different ways to create a histological activity grade, for example the

Geboes scale (Geboes et al., 2000) and the Riley index (Riley et al., 1991). Although occasionally helpful in UC, for Crohn's, both diagnosis and disease activity are far less dependent on histology and although certain histological features are helpful to predict disease activity and progress, there is no expert consensus on using these to grade activity (Stange et al., 2006, Mowat et al., 2011).

Histological activity is rarely used as a grading tool in clinical practice and has not yet been used as an endpoint in clinical trials. Mucosal inflammatory activity is defined mainly on the macroscopic appearance which correlates with sustained remission as demonstrated in the landmark trials, Activity in Ulcerative Colitis Trial 1 and 2 (ACT 1 and 2). In this trial Rutgeerts et al. (2005) showed that macroscopic mucosal healing after 8 weeks of anti-TNF- $\alpha$  therapy correlated with a 4 fold increase in symptomatic remission at 30 weeks, but did not venture to compare microscopic healing.

In practice the results from histology is most often indeterminate and of little help other than ruling out alternative diagnoses and confirming inflammation.

## 1.2 The immune system

### 1.2.1 Introduction

The immune system is a collection of biological structures and processes which defend the human body against an almost infinite array of harmful substances<sup>3</sup>.

An effective immune system achieves a balance between a swift and aggressive response to injurious substances, controlled and timely reduction in that response, a recognition and tolerance to self-antigens and creation of long term immunity. A defective immune response has been strongly associated with many human diseases including many types of cancer (Ohm and Carbone, 2002), chronic inflammatory diseases such as IBD and arthritis (Baumgart and Carding, 2007), many metabolic diseases including obesity and nutrient deficiencies (Engstrom et al., 2003), autoimmune conditions such as diabetes, thyroid dysfunction and coeliac disease (Davidson and Diamond, 2001), hypersensitivity reactions such as anaphylaxis, immunodeficiency such as HIV and many more (Iborra et al., 2012).

Our immune system has been evolving for approximately 3 billion years since the most primitive life forms developed a defence against invasion by foreign substances. Some features of these primitive defences remain and include the defensins and complement cascades, however over time pathogens have evolved to circumvent these crude defences, and so our immune system has evolved also. The modern human immune system can be subdivided into 3 main mechanisms of defence; a physical barrier against invasion, the swift non-specific response of the innate immune system against generic pathogenic components and the slower adaptive immune system which is capable of providing a targeted response and the development of lasting immunity.

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<sup>3</sup> This includes infective pathogens such as microbes, parasites and viruses, autologous substances and cells which left unchecked have the potential to become cancerous, and other organic molecules and chemicals.

## 1.2.2 The barrier

### 1.2.2.1 Introduction

The first line of defence against any pathogen is to prevent its admission into the human body. Access is prevented initially by a series of mechanical, chemical and biological barriers. The gastrointestinal tract has several cleansing mechanisms which are commonly enrolled to rid the gastrointestinal tract of harmful substances and pathogens including the actions of vomiting and diarrhoea. In addition, orally consumed pathogens are met by a hostile environment including the chemical barriers of gastric acid, bile and pancreatic secretions followed closely by the biological inhibition of foreign bacteria by a balanced growth of commensal flora in the small bowel. If pathogens manage to survive these defences, they are then kept within the lumen of the intestine by a single layer of columnar epithelial cells (EC) coated with a protective mucus layer collectively known as the epithelial cell barrier Figure 1.

### 1.2.2.2 The epithelial cell barrier

The function of the epithelial cell barrier can be divided into 2 overlapping areas; the first is its role as a physical barrier separating the luminal contents from the body and the second are the role it plays in a variety of immune functions.

The colon contains over 5500 different species of bacteria (Dethlefsen et al., 2008) at a total concentration of between  $10^{11}$  and  $10^{12}$  microbial cells per gram of luminal contents. These bacteria perform some crucial metabolic and protective roles, however some are potentially harmful pathogens and must be prevented from penetrating through the mucosal barrier (Guarner and Malagelada, 2003).

Mucus is the first line of defence and lines the entire GI tract. It is thickest in the colon, where it serves as a permeable gel through which essential macromolecules and nutritional substances can pass but acts as a protective membrane against larger molecules, e.g. digestive enzymes, toxins and bacteria. Colonic mucus is composed of many defensive factors such as antimicrobial peptides, defensins and the immunoglobulins IgA and IgG.

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The integrity of the epithelial cell barrier is maintained by a layer of polarised epithelial cells which are adhered together by cell to cell junctional proteins, Figure 1. These proteins are modulated by a wide range of cytokines and growth factors and allow selective passage of macromolecules, nutritional substances and specialised dendritic cells (DC).

Epithelial cells function as more than just a physical barrier, it is becoming increasingly recognised that epithelial cells influence the outcome of innate and adaptive immune responses through expression of factors such as thymic stromal lymphopoietin (TSLP) which influences DC function to favour a  $T_H2$  mediated inflammatory response. TSLP is discussed in more depth in Chapter 6.

Dendritic cells are allowed to bridge the epithelial tight junctions to sample the luminal contents. Luminal sampling by DCs promote the development of immunity against potentially harmful luminal antigens, whilst developing tolerance to self and innocuous substances to prevent unrestrained activation of the immune system.

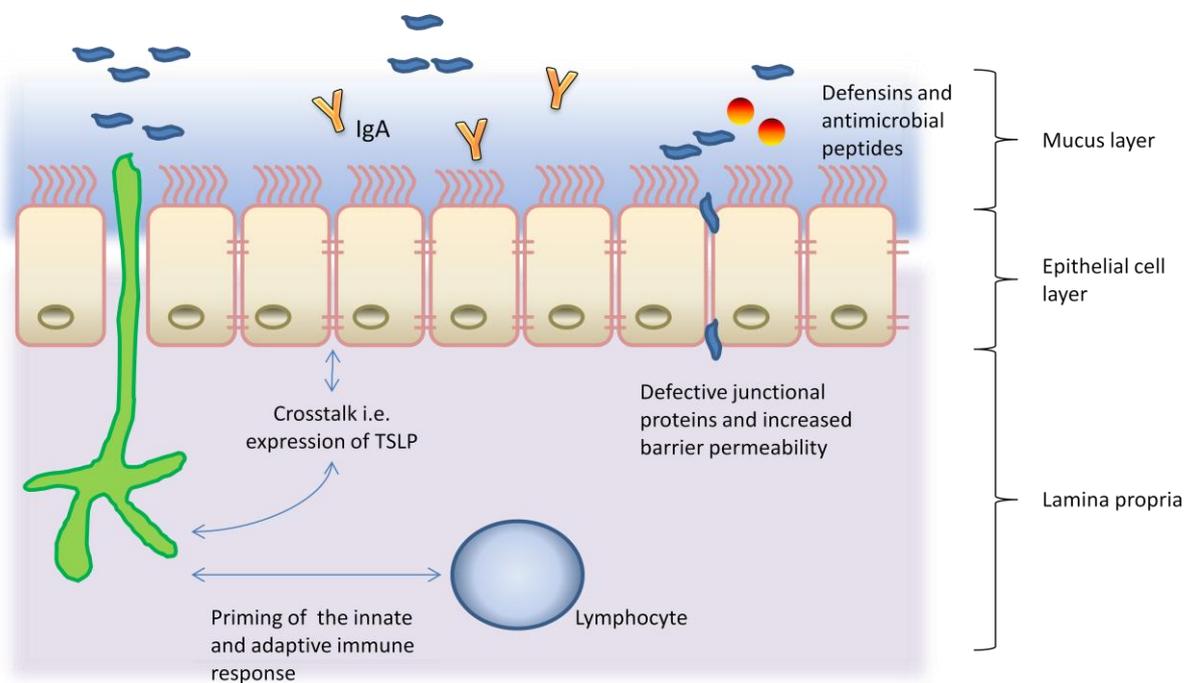


Figure 1. The colonic epithelial cell barrier

## 1.2.3 Innate immune system

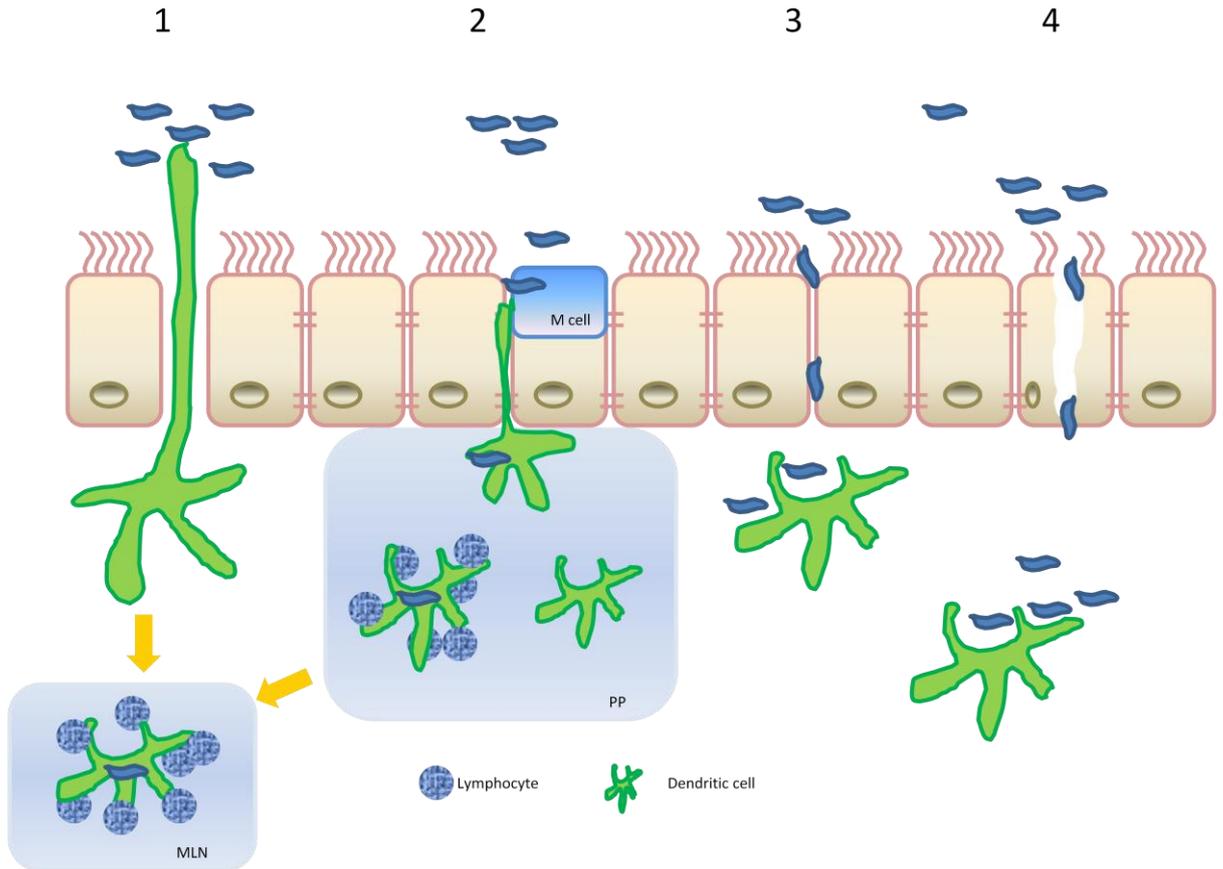
### 1.2.3.1 Introduction

In terms of evolution, the innate immune response is the oldest part of the immune system and has evolved to prevent free growth of bacteria. The innate immune system leads to a fast but non-specific response against generic components of invading organisms and injured cells. These generic components are molecular motifs that create recognisable patterns called pathogen associated molecular patterns (PAMPs).

The major functions of the innate immune system within the GI tract are to:

1. Recruit immune cells to the site of invasion or injury.
2. Activate the complement cascade.
3. Identify and remove foreign substances via phagocytosis.
4. Activate the adaptive immune system through antigen presentation and co-stimulatory signals.

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**Figure 2. Dendritic cell sampling of luminal contents.** Dendritic cell exposure to luminal antigens occurs in 4 ways; Active luminal sampling (1 and 2) and active sampling following bacterial invasion through the epithelial barrier (3 and 4). 1. DCs reach through the epithelial barrier and sample directly from the lumen. 2. M cells are specialised cells that mediate uptake of bacteria in intestinal lymphoid tissue. 3. The bacteria reach the lamina propria by direct invasion through the epithelial barrier due to increased permeability. 4. Tissue damage. After antigen encounter, DCs travel in local lymphoid structures such as Peyer's patches (PP) and toward the draining mesenteric lymph nodes (MLN) to initiate or maintain T and B lymphocyte cell immune responses.

The innate immune system consists of many cell types each with their own unique characteristics and functions. Mast cells, macrophages and dendritic cells are specific types of phagocytic and antigen presenting cells (APC) that reside in their inactive state within the interface between the outside world and body tissues, in the GI tract this is the lamina propria of the intestinal mucosa. They are constantly sampling the local environment for antigens that have breached the epithelial cell barrier through increased permeability or injury. In addition, DCs have the unique ability to sample the luminal contents of the intestine by extending their dendrites

through the epithelial cell barrier whilst maintaining barrier integrity through the expression of junctional proteins; occludin, claudin 1 and zonula occludens 1 (Rescigno et al., 2001), Figure 2.

Through the process of luminal sampling the DCs of the innate immune system are able to present antigens to the adaptive immune system to develop a specific immunity against certain antigens that are potentially harmful, and tolerance against other antigens which are helpful to survival and the maintenance of epithelial homeostasis. This tolerance prevents the unwanted activation of the immune response to harmless and / or helpful commensal bacteria.

### 1.2.3.2 Pattern recognition receptors

A vital function of the innate immune system is the ability to distinguish between potentially harmful antigens and products of normal cellular processes. The molecules responsible for this function are pattern recognition receptors (PRRs), which include; the membrane bound Toll like receptor (TLR) family which recognise PAMPs on the cell surface or within extracellular compartments, the Nod like receptor (NLR) family which recognise intracellular PAMPs and the recently identified retinoic acid inducible gene 1 (RIG-1) and C-type lectin receptors.

### 1.2.3.3 Toll like receptors

In 1994 the first TLR in humans was reported (Nomura et al., 1994), to date another 12 have been identified, TLR1, 2, 4, 5 and 6 are located on the cell surface and TLR3, 7, 8, 9,12 (possibly) and 13 are localized to the endosomal / lysosomal compartment. Toll like receptors are characterised by an extracellular domain which consists of a leucine rich repeat (LRR), a single transmembrane domain and a cytoplasmic domain called Toll/interleukin 1 receptor (TIR) domain, Figure 3.

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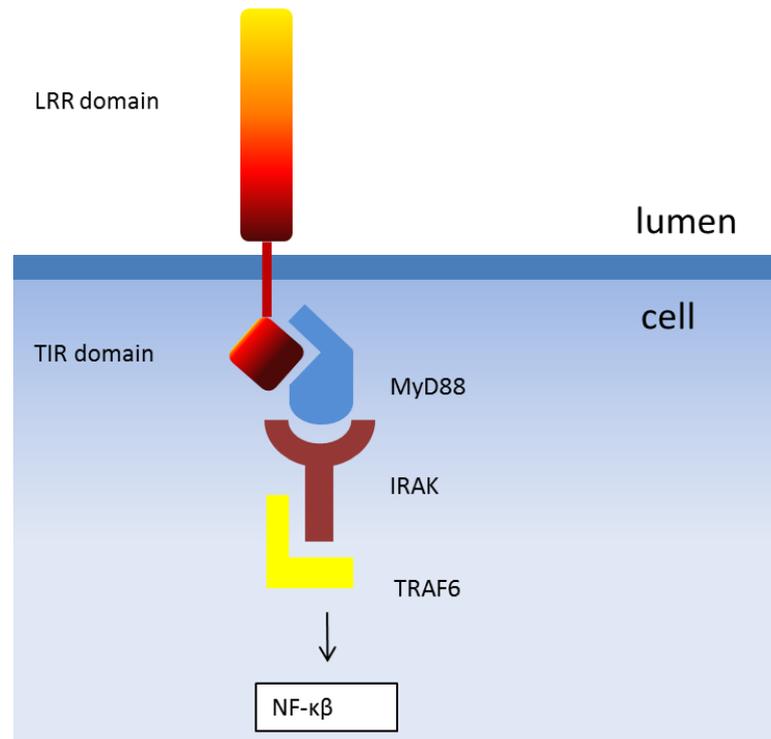


Figure 3. MyD88 mediated activation of the Toll like receptor pathway

The LRR domain is thought to be the ligand sensing motif which is able to identify conserved microbial PAMPs or other ligands. TLR4 is perhaps the most best known TLR and has been shown to recognise a generic feature of bacterial cell wall, the lipopolysaccharide (LPS) (Poltorak et al., 1998). Activation of the TLR4 signalling pathway originates from the cytoplasmic Toll/IL-1 receptor (TIR) domain that associates with a TIR domain-containing adaptor, MyD88. Upon stimulation of TLR4 by LPS, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs through interaction of the death domains of both molecules. IRAK activated by phosphorylation then associates with TRAF6 leading to activation of JNK and the transcription factor NF- $\kappa$ B and IFN- $\gamma$ . The final result of the PRR pathway is activation of the inflammatory cytokines IL-6, IL-1 and TNF- $\alpha$ .

Since the observation that NF- $\kappa$ B is expressed, although delayed, after TLR4 stimulation in MyD88 deficient macrophages (Kawai et al., 1999), at least three MyD88 independent pathways have been identified (Takeda and Akira, 2005). These include other TIR domain containing adaptors, TIR domain containing adaptor inducing IFN- $\gamma$  (Yamamoto et al., 2002, Hoebe et al., 2003), TRIF related

adaptor molecules (Yamamoto et al., 2003), and IRF-3 (Kawai et al., 2001). The roles of these MyD88 independent pathways are likely to represent an evolutionary “redundancy” measure to protect against potentially fatal consequences of a defective MyD88 pathway.

#### 1.2.3.4 NOD like receptors

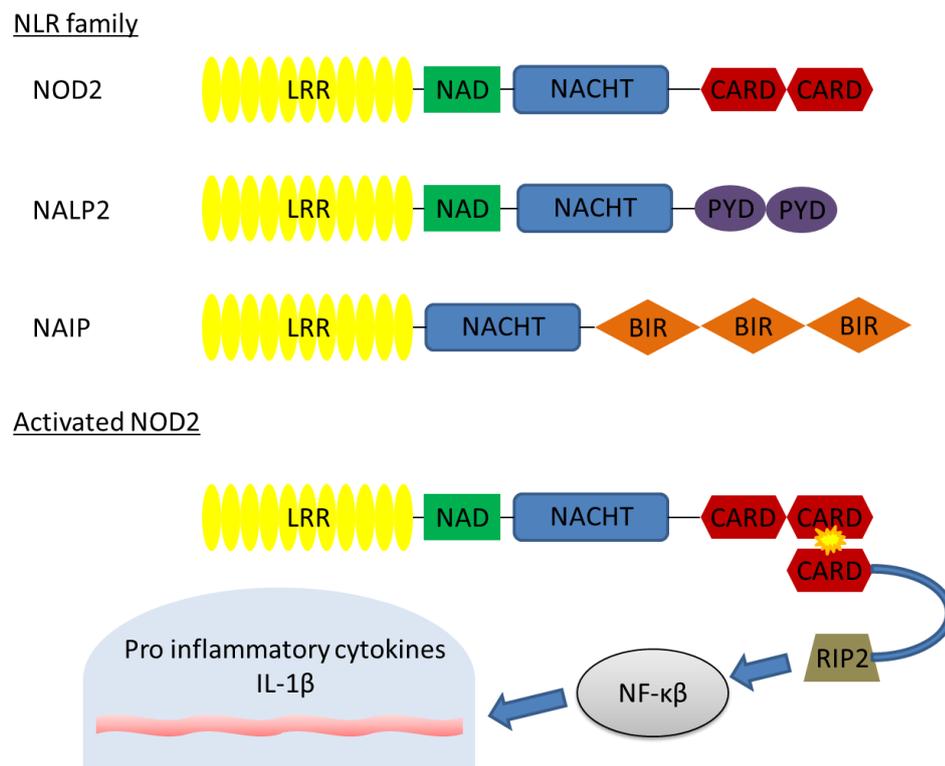


Figure 4. The NLR family and activation of NOD2

The largest family of intercellular PRRs is the nucleotide-binding oligomerization domain [NOD] like receptors (NLRs). The NLRs include; NALP(1-14), NOD (1-5), CIITA, IPAF and NAIP (Martinon and Tschopp, 2005). Three domains characterise the NLRs; a LRR domain similar to TLRs, which recognises PAMPs, a NACHT domain which is essential for NLR activation, and the N-terminal effector domain. Oligomerization of the NACHT domain is essential for the signal mediated by the N-terminal effector domain which is either a pyrin domain (PYD), caspase



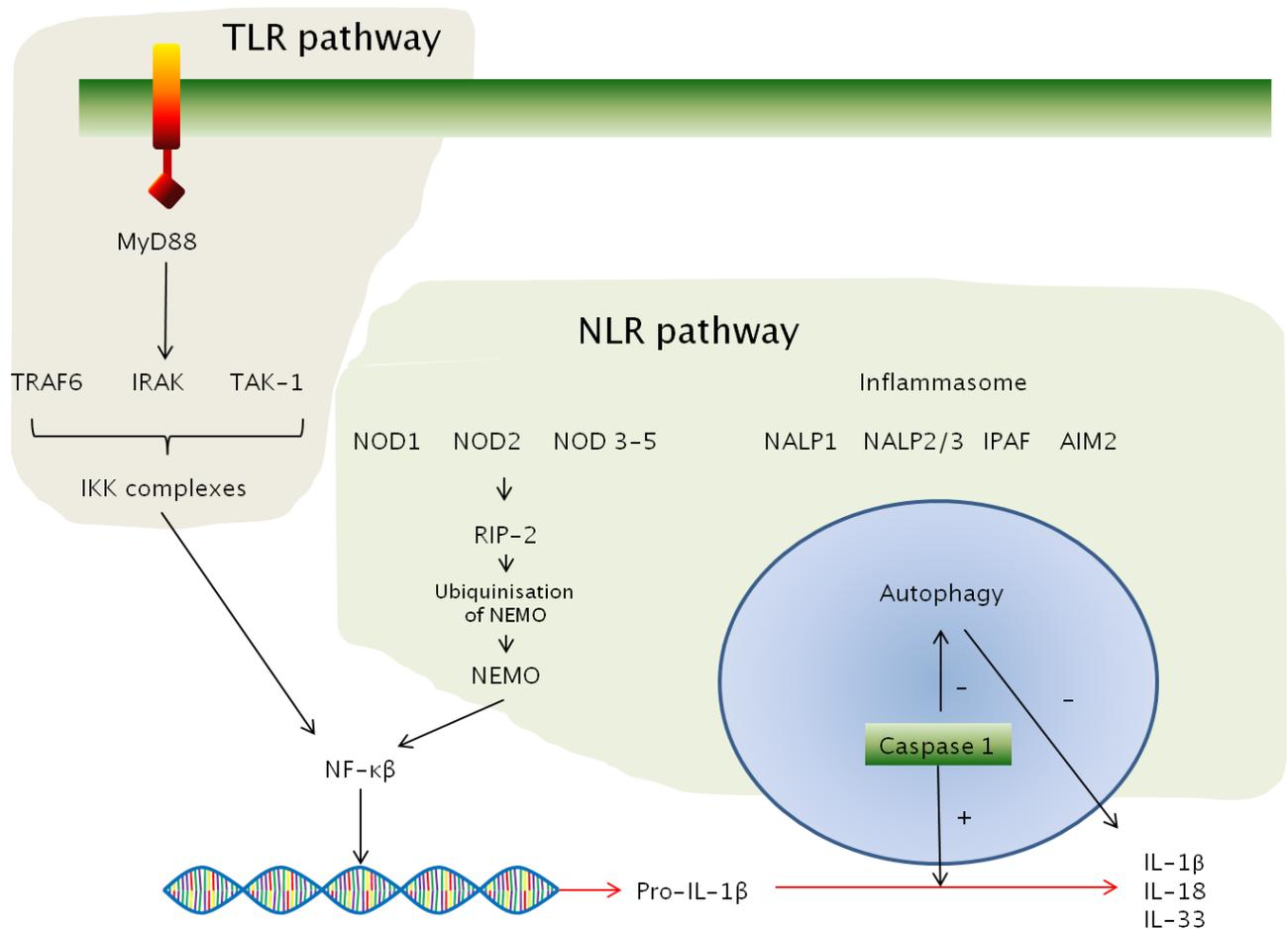


Figure 6. The interaction of the TLR and NLR pathways and the production of IL-1 $\beta$ , IL-18 and IL-33.

The main functions of NLRs are to detect intracellular PAMPs and trigger the expression of the inflammatory cytokines IL-1 $\beta$ , IL-18 and possibly IL-33. The most potent of the inflammatory cytokines is IL-1 $\beta$  which is an important mediator of many immune responses and cellular activities. The expression of IL-1 $\beta$  and IL-18 is complex and occurs by a slightly different mechanism depending on the NLR which has been activated and the cell type in which it is occurring. There is a common first step; the expression of pro-IL-1 $\beta$ , the inactive precursor molecule of IL-1 $\beta$ . The production of Pro-IL-1 $\beta$  is mediated by NF- $\kappa$ B through stimulation and activation of TLRs, although the activation of any PRR can produce NF- $\kappa$ B, the

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activation by TLRs has become known as “priming”. The second step depends on which NLR is activated, Figure 6.

The function of TLR and NLR pathways interact synergistically. This symbiotic relationship protects against the inappropriate activation of inflammatory processes by a single pathway, for example, inflammasome activation of caspase-1 alone without priming (activation of NF- $\kappa$ B by the TLR pathway) does not lead to increased IL-1 $\beta$  production despite increased levels of active caspase 1 (Mariathasan and Monack, 2007). However, there are exceptions, in a review by Netea et al. (2008) it is explained that under certain environmental situations monocytes are capable of releasing active IL-1 $\beta$  upon stimulation of TLR ligands alone, in contrast to macrophages which do require TLR and NLR stimulation, an adaptation that is likely to reflect certain environmental situations. None the less combined TLR and NLR activation in monocytes enhances cytokine production more than TLR activation alone.

### 1.2.4 Adaptive immune system

#### 1.2.4.1 Introduction

In contrast to the swift but non-specific inflammatory response of the innate immune system, the adaptive immune system provides a slower but targeted response to maximise the clearance of specific pathogens from the body. Other important characteristics of the adaptive immune system include the ability to differentiate self from non-self and the ability to create immunological memory to prevent subsequent infections.

Adaptive immunity is triggered through the process of antigen presentation. Although all nucleated cells are capable of presenting antigen only dendritic cells, B lymphocytes and macrophages are termed antigen presenting cells because of the specialised receptors that allow activation of T lymphocytes. Antigen presenting cells engulf pathogens following recognition of PAMPs by their PRRs; this is the immediate response of the innate immune system. Within the APCs enzymes split larger pathogens into smaller particles which can then be displayed as antigens on the cell surface of the APCs by major histocompatibility complexes

(MHCs). Presentation of the antigen / MHC complex on the cell surface of APCs then activates the effector cells of the adaptive immune system. Effector cells are the T and B lymphocytes.

#### 1.2.4.2 B Lymphocytes

The primary role of the B lymphocyte (also known as B cell) is to mastermind the humoral response through creation and release into the systemic circulation, and / or local environment, specific antibodies (immunoglobulins) (LeBien and Tedder, 2008). Antibodies bind to specific antigens which are either targeted randomly through VDJ recombination during B cell maturation or through clonal expansion following B cell activation. The antigen-antibody complex causes a number of effects; disruption of the invading pathogen by interruption of its cell wall integrity, the prevention of pathogen binding to host cells, marking pathogens for destruction by the phagocytic cells of the innate immune system, activation of the complement cascade and antigen clearance via the spleen or liver.

Each B cell has a unique receptor protein called the B cell receptor (BCR), this is an immunoglobulin which an enormous degree of diversity. The immunoglobulin contains a heavy and light domain split into 3 sections, V, D and J. Through a random, and error prone, process of VDJ recombination in which random changes in the antibody gene locus occur during B cell maturation, a unique BCR for each B cell clone is created. This process of random BCR creation ensures that B cells are able to recognise and process an almost limitless number of antigens.

Dysfunctional B cells and those displaying self-recognising BCRs are removed at an early developmental stage by mechanisms including clonal deletion, receptor editing and anergy. Mature but naïve B cells which express both IgM and IgD immunoglobulins are released into the circulation and migrate to the secondary lymphoid organs of the periphery. Upon recognition of its cognate antigen by the BCR, B cells are able to engulf the pathogen and present processed peptides in the cell membrane with both MHC class 1 and 2 complexes. The activated B cell then requires a further step to produce antibodies, this can occur through a T lymphocyte dependent or independent mechanism (Vale and Schroeder, 2010).

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The typical T lymphocyte (also known as the T cell) activation pathway is T cell dependent and mainly occurs in the germinal centres of lymphoid tissues. B cells present antigen / MHC complexes on their cell surface to CD4<sup>+</sup> helper T cells (T<sub>H</sub>), or CD8<sup>+</sup> cytotoxic T cells. Through the production of a cytokine milieu, B cells are stimulated to do a number of different things; proliferate in a process called clonal expansion, class switch recombination (a process where the secreted immunoglobulin is IgG, or IgE rather than IgM), differentiate into short lived plasma cells which release antibody or differentiate into long lived memory B cells.

The T cell independent mechanism is critical and allows the B cell to recognise antigens that are not recognised by T cells such as DNA or polysaccharides. T cell independent activation allows class switching and production of IgG immunoglobulins independent of the peripheral lymphoid tissues.

T cells constitute the largest proportion of lymphocytes in the GI tract mucosa, with a distribution which is variable along the length of the bowel and between layers and influenced by local requirements and conditions (Cheroutre et al., 2011). Sub families of T cells have specific roles in the cell mediated response.

### 1.2.4.3 T lymphocytes

The cell-mediated immune response is lead by the T lymphocyte and encompasses a highly heterogeneous group of immune cells, Figure 7. T cells are characterised by the T cell receptor (TCR). The combination of CD3 and TCR is known as the TCR complex, CD3 is a transmembrane protein whose activation is essential for TCR signalling. The TCR is responsible for recognising antigen that is bound to MHC complexes on the surface of other cells. It is a heterodimer consisting of 2 protein chains, in 95% of T cells, this is the  $\alpha/\beta$  chain, in the other 5% it is the  $\gamma/\delta$  chain. Each chain of the TCR is a member of the immunoglobulin superfamily and contains a constant domain, which sits across the cell membrane, and a variable domain which is formed by VDJ recombination during T cell maturation. The randomness of VDJ recombination ensures that the TCR recognises a great diversity of processed and presented antigen.

The cell mediated immune response involves the activation of cells that directly phagocytose and destroy pathogens such as macrophages and natural killer cells, and the activation of cytotoxic T cells which induce apoptosis in cells that are displaying antigen on their surface, and the stimulation of cells to secrete a variety of cytokines that themselves influence the defensive pathways of the innate and adaptive immune system.

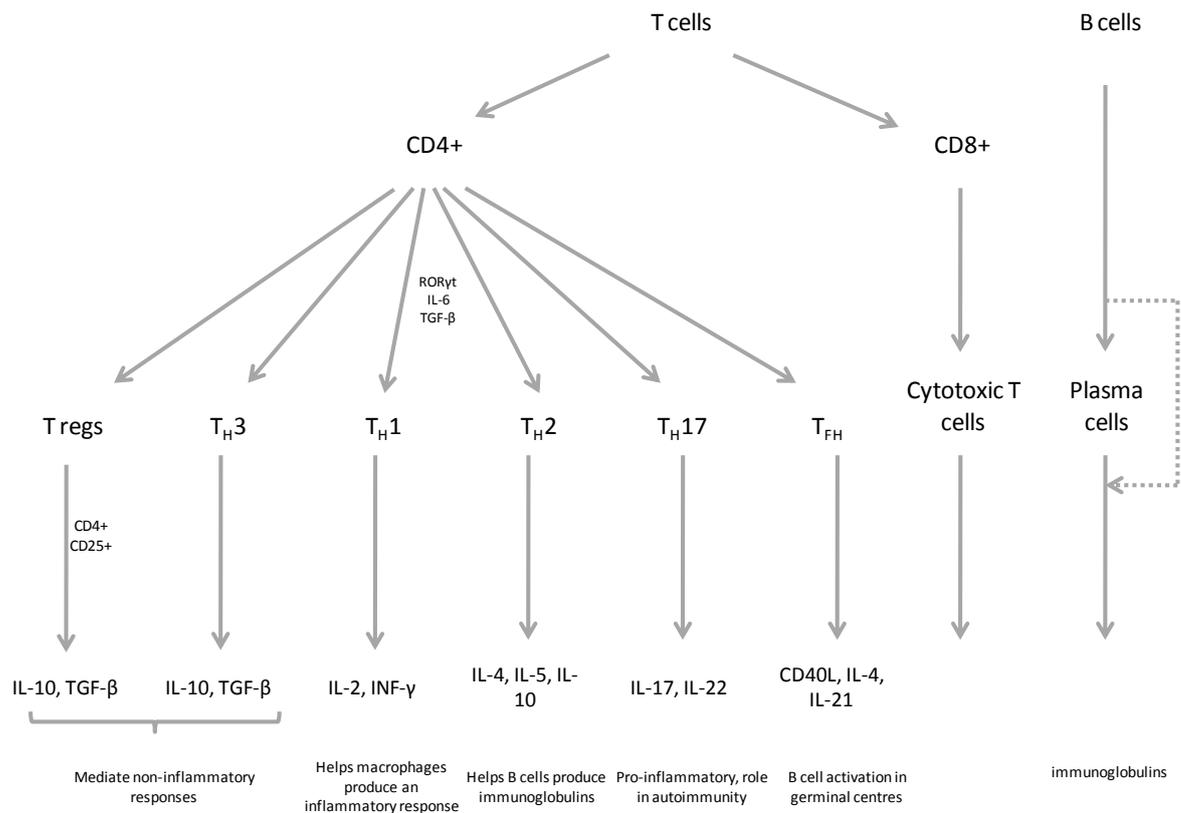


Figure 7. The differentiation of precursor cells into the effector cells of the adaptive immune system

Along with the TCR complex the antigen / MHC complex will bind to specific co-receptors on the surface of T cells, for T helper cells this is the CD4 co-receptor specific to MHC class 2 molecules and for cytotoxic T cells it is the CD8 co-receptor specific for the MHC class 1 molecule. The addition of co-receptor binding is to enhance and activate a chain of specific intercellular cascades. Other important membrane proteins include CD45 which has a phosphatase on its

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intracellular section which helps to activate pathways within the cytosol and CD28 which is the receptor for CD80 a protein which is up-regulated on the surface of activated APCs, and acts as a failsafe mechanism. Activation of CD28 by CD80 is necessary for the T cell to avoid becoming anergic, this ensures the T cell is only activating against those foreign antigens presented on APCs rather than self-antigens present in the local environment. The role of CD45 is not entirely known but conformational changes within the CD45 molecule help to indicate the state of T cell activation and maturation, (CD45RA<sup>+</sup> has not been activated, CD45RO<sup>+</sup> has been activated).

### 1.2.4.3.1 CD4<sup>+</sup> T helper cells

Included within the T helper cell family are; T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>17 lymphocytes, all express the surface protein CD4, which during maturation determines the eventual affinity to class 2 MHC molecules. T helper cells orchestrate the immune response by activating and directing other cells of the immune system, the effect of their dysfunction or absence is demonstrated by a number of diseases, many of which are fatal, the most well-known is the Acquired Immune Deficiency Syndrome following inactivation of CD4<sup>+</sup> cells by the human immunodeficiency virus.

When the TCR complex and co-receptors are activated the naïve T cell, T<sub>H</sub>0, is capable of proliferation and clonal expansion through release of T cell growth factor, IL-2 and IL-2R. Depending on the cytokine milieu T<sub>H</sub>0 cells then differentiate, IFN- $\gamma$  drives a T<sub>H</sub>1 cell proliferation whilst IL-10 and IL-4 inhibit it. On the other hand IL-4 drives a T<sub>H</sub>2 cell proliferation whilst IFN- $\gamma$  inhibits it. Determining the balance between T<sub>H</sub>1 and T<sub>H</sub>2 is crucial as the different T cell sub-families generate a different pattern of immune response. A T<sub>H</sub>1 response maximises the efficacy of the cellular immune response particularly macrophages and CD8<sup>+</sup> cells and is associated with the production of cytokines INF- $\gamma$  and TNF- $\alpha$ . A T<sub>H</sub>2 response stimulates the B cells into proliferation, class switching and antibody production, and is associated with the production of IL-4, IL-5, IL-6, IL-10 and IL-13.

T<sub>H</sub>17 are a distinct sub family of T helper cells, discovered in 2005 (Harrington et al., 2005) they are thought to play a vital role in pro-inflammatory pathways at epithelial and mucosal barriers. The differentiation of T<sub>H</sub>0 cells into T<sub>H</sub>17 cells has

been extensively studied (mostly in mice) with conflicting results. In humans a combination of TGF- $\beta$ , IL-1 and IL-23 drive the differentiation of T<sub>H</sub>0 towards the T<sub>H</sub>17 cell lineage, whereas INF- $\gamma$  and IL-4 drive the differentiation of T<sub>H</sub>0 away from T<sub>H</sub>17 towards the T<sub>H</sub>1 and T<sub>H</sub>2 lineages. An important process shown in animal models has been the influence of TGF- $\beta$  and IL-6 on the differentiation of T<sub>H</sub>0 cells into either regulatory T cells (T<sub>regs</sub>) or T<sub>H</sub>17 cells, thus influencing either a pro- or anti-inflammatory state. Furthermore a cytokine produced by mature T<sub>H</sub>17 cells, IL-21 also drives an IL-23 independent differentiation from T<sub>H</sub>0 cell precursors. Once differentiated, IL-23 is also associated with the expansion of established T<sub>H</sub>17 populations, perpetuating the inflammatory response.

Transcription factors that play a role in T<sub>H</sub>17 differentiation are STAT3 (Liu et al., 2008), IRF4 (Huber et al., 2008), ROR $\alpha$  and ROR $\gamma$  (Yang et al., 2008), and ROR $\gamma$ t (Ivanov et al., 2006). In reality there are several proposed and independent pathways through which T<sub>H</sub>17 cells are differentiated from T<sub>H</sub>0 cells or expanded and activated.

The key cytokine product of T<sub>H</sub>17 cells is IL-17 which has a number of functions including recruitment and activation of neutrophils and macrophages, and induction of defensins by epithelial cells. Other effector cytokines of T<sub>H</sub>17 cells include, IL-21, IL-23, although IL-17 is the predominant cytokine. T<sub>H</sub>17 cells, through the production of IL-17, also produce TNF- $\alpha$ , IL-1 $\beta$ , IL-6, G-CSF, MIP-2, CXCL1, CXCL2 and CXCL8. Although not directly involved in inflammation, T<sub>H</sub>17 cells orchestrate other cells that are capable of inappropriate tissue damage. Dysfunction of T<sub>H</sub>17 cells is thought to play a major role in many autoimmune diseases and immunodeficiency disorders.

### 1.2.4.3.2 Regulatory T cells

Regulatory T cells (T<sub>regs</sub>) are characterised by the surface expression of both CD4 and CD25, and the transcription factor Forkhead box p3 (FoxP3) which is crucial for their development and function. Regulatory T cells play an important role in suppressing autoimmunity and controlling inflammation by suppressing the proliferation of pro-inflammatory cells and the secretion of anti-inflammatory cytokines. Regulatory T cell dysfunction is clinically expressed in the human

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disease immunodysregulation polyendocrinopathy and enteropathy X-linked syndrome (IPEX) which is a result of a point mutation in the FOXP3 gene leading to dysfunctional FoxP3.

The generation of  $T_{regs}$  from  $T_H0$  cells is driven by TGF- $\beta$  in a similar way to  $T_H17$  cells, however the expression of the pro-inflammatory cytokine IL-6 appears to inhibit FoxP3 and therefore the production of  $T_{regs}$  by driving differentiation towards the  $T_H17$  lineage (Kimura and Kishimoto, 2010).

Regulatory T cells infer tolerance and inflammatory suppression via 2 core mechanisms, bystander suppression and infectious tolerance. Bystander suppression is the non-specific suppression of activated effector cells, and infectious tolerance occurs when a new population of  $T_{regs}$  with suppressive activities against a specific antigen are created. The molecular mechanisms through which  $T_{regs}$  exert their influence are complex and increasing in number. In a review by Tang et al. (2008), 13 separate molecular mechanisms are reported in which  $T_{regs}$  function, they are grouped into; those mechanisms that influence other cells, those mechanisms that alter APC activity and those mechanisms that alter the secretion of suppressor molecules. The 13 molecular mechanisms included the expression of cytokines IL-2, IL-10, IL-35, TGF- $\beta$ , IFN- $\gamma$ , the enzymes Granzyme B and Perforin and receptors CTLA-4 and CD25.

Central to the functional effect of  $T_{regs}$  is the transcription factor FoxP3. FoxP3 directly increases CTLA-4 and CD25 and decreases IL-2 expression. Regulatory T cells constitutionally express high levels of membrane bound CTLA-4 which acts as a negative regulator of the CD80 / CD86 protein on the surface of APCs. The inhibition of CD80 / CD86 expression on developing dendritic cells in germinal centres and the peripheral down regulation of CD80 / CD86 on mature DCs confers a tolerance to specific antigens.

Constitutive expression of high levels of CD25 (IL-2 receptor), and decreased expression of IL-2 ensure that  $T_{regs}$  are highly reliant on exogenous IL-2 production for their survival. The absorption of IL-2 depletes the local environment and therefore reduces the availability of the pro-inflammatory cytokine IL-2 to other

effector cells. Deficiency of IL-2 or CD25 leads to autoimmunity or inflammation akin to conditions directly caused by  $T_{reg}$  deficiency.

Disrupting  $T_{reg}$  function in experimental models can induce a pro-inflammatory effect, however translating this into an effective treatment for autoimmune and inflammatory conditions by enhancing the suppressive effects of  $T_{regs}$  has not been shown, and is hampered by amongst other things, the phenomenon of “plasticity” where by T cells are able to change their phenotype, in the case of  $T_{regs}$ , from suppressive to pro-inflammatory phenotype depending on the conditions (Zhou et al., 2009).

#### 1.2.4.3.3 CD8<sup>+</sup> cytotoxic T cells

CD8<sup>+</sup> cytotoxic T cells are a family of T lymphocytes capable of inducing the death of infected cells. Along with a TCR they constitutional possess the CD8 membrane glycoprotein that binds to the non-variable domain of a MHC class 1 molecule / antigen complex.

Activation of CD8<sup>+</sup> T cells is similar to that of CD4<sup>+</sup>. Firstly the APC presents a processed peptide antigen with a MHC class 1 molecule, this is recognised and bound by the TCR and further stabilised by the addition of the CD8 binding. The co-receptor CD28 then binds with CD80 or CD86 expressed on the surface of the APC. Once activated, under the influence of IL-2, the CD8<sup>+</sup> T cell undergoes clonal expansion for the specific antigen presented by the APC.

Activated CD8<sup>+</sup> T cells induce apoptosis in targeted cells in a number of ways, although the primary mechanism is through the initial release of perforins to disrupt the cell membrane and allow the access of various granzymes which activate the intracellular caspase cascade resulting in apoptosis.

## 1.3 The immune system and IBD

### 1.3.1 Introduction

It is generally accepted that a deregulated immune response lies at the heart of IBD, although attempts to elicit the nature of this have been hampered by the complex interplay of causative factors, immune response, disease definition and experimental interpretation.

Over the last 30 years the major advances in IBD have centred on the principle that IBD is the result of an abnormally polarised T cell response (Fuss, 2008, Neurath et al., 2002). This is encompassed in the "*T<sub>H</sub>1-T<sub>H</sub>2 paradigm*", first described by Mosmann and Coffman (1989) from observations on experimental inflammation. The T<sub>H</sub>1-T<sub>H</sub>2 paradigm proposes that under certain conditions naïve T cells are driven to differentiate either into T<sub>H</sub>1 or T<sub>H</sub>2 effector T cells. The abnormally enhanced proliferation and function of either T<sub>H</sub>1 or T<sub>H</sub>2 cells will drive a distinct pattern of inflammation and cytokine profile which in the case of GI tract inflammation produces an inflammatory pattern characteristic for either Crohn's or UC.

The "*T<sub>H</sub>1-T<sub>H</sub>2 paradigm*" describes Crohn's as a predominantly T<sub>H</sub>1 cell mediated inflammatory response with the associated excess of cytokines IL-12, IL-23, IFN- $\gamma$ , IL-17 and TNF- $\alpha$ , and UC as a predominantly T<sub>H</sub>2 cell response with an excess of the cytokines IL-4, IL-5 and IL-13 (Strober et al., 2007, Fuss, 2008).

The goal of research is to define IBD in terms of a solitary dysfunctional pathway, although recently described T cell lineages, such as T<sub>H</sub>17, T<sub>H</sub>3, T<sub>regs</sub>, NK cells, and cytokines such as IL-23 and IL-17, also play a crucial role in IBD and do not fit comfortably into the T<sub>H</sub>1-T<sub>H</sub>2 paradigm. It is therefore likely that the disease initiation and perpetuation is the end result of multiple dysfunctional elements of numerous pathways, a "*multi-hit*" idea, a concept that is supported by emerging genetic data, see chapter 1.3.7.1.

Further to the "multi-hit" idea, attempts to find a unifying causative factor have been disappointing, and although specific cytokine blocking agents (e.g. TNF- $\alpha$  blockers) are now available their long term efficacy is disappointing, reminding us

that IBD is almost certainly a multi factorial disease within an immune system which is rapidly able to adapt and overcome invasion and external intervention.

All aspects of the immune system have been implicated in the aetiology and perpetuation of IBD although through the use of clinical observation, animal models, engineered cell lines and the limited use of unaltered (either by treatment or experimental design) human IBD tissue broad themes are emerging, Table 10.

	mechanism	Notes
Dysbiosis	Host - microbial homeostasis	Review of treatments which alter commensal bacteria and their potential benefit in IBD (Kanauchi et al., 2003)  Review of trends in the literature in favour of Dysbiosis as a cause of IBD (Kaur et al., 2011)
	Specific antigen presentation	Not known yet
	Specific infectious agents	None yet identified, no clinical benefit has been demonstrated following the treatment of proposed infectious agents such as E coli.
autoimmunity	Loss of tolerance	A review of tolerance in human IBD (Maul and Duchmann, 2008)
Barrier dysfunction	Mucus production	Decreased mucus is seen in UC (Cope et al., 1988)  Muc2-/- deficiency in mice results in spontaneous colitis (Velcich et al., 2002)
	Epithelial barrier dysfunction	Review of barrier dysfunction focusing on epithelial cell leakiness, the multi-functionality of tight junctions and microbiome in IBD (Laukoetter et al., 2008)  Increasing attention to mucosal healing as an important endpoint in trials and treatment, reviewed by Neurath and Travis (2012).  Increased micro-erosions and epithelial cell permeability in IBD seen by confocal laser endomicroscopy (Kiesslich et al., 2012).
	Tight junction leakiness	Differential expression of Claudin 2,3 and 4 in IBD (Prasad et al., 2005)
Innate immunity	Dysfunction of macrophages	miR-192 influences the expression of MIP-2 $\alpha$ in HT29 cell lines (Wu et al., 2008)
	Dysfunction of dendritic cells	DCs exposed to mucosal epithelial cell microenvironment (TGF- $\beta$ and retinoic acid) promote regulatory T cell differentiation and a tolerogenic cytokine environment (Iliev et al., 2009)

Table continued overleaf

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	mechanism	Notes
Adaptive immunity	Dysfunction of T <sub>regs</sub>	Decreased ratio of T <sub>regs</sub> : T <sub>H</sub> 17 cells in peripheral blood in IBD (Eastaff-Leung et al., 2009)  Increased in the mucosa during active IBD, but not as much as other types of inflammation (Maul et al., 2005)  Dysfunction in experimental models leads to colitis (Asseman et al., 2000)
	T cell dysfunction	Overall, total numbers of mucosal T cells is probably raised in IBD compared with health but probably not as high as other inflammatory conditions. Results depend on experimental method. No imbalance of mucosal CD4 / CD8 T cells in IBD (Selby et al., 1984) Intraepithelial CD4 : CD8 ratio increased in UC but not Crohn's (Caballero et al., 1995), however it is the cytokine imbalance which is more important suggestive of functional imbalance rather than total number of cells.
	Cytokine imbalance	The cornerstone of potential therapeutic targets in IBD with over 30 therapies in development most of which target specific cytokines (Danese, 2012). Review of prominent cytokines by (Sanchez-Munoz et al., 2008).  IBD susceptibility alleles include IL-17A and IL-23R, both strongly involved in T <sub>H</sub> 17 cell activation and activity in Korean population (Kim et al., 2011), and multiple other alleles including IL-23R, IL-12B and IL-10 identified by GWAS in white populations (Lees et al., 2011).
Others	miRNAs (see section 1.4.3 MicroRNA in IBD)	Seminal paper on the miRNA signature in the colonic mucosa of patients with UC (Wu et al., 2008)  Review of miRNA dysfunction in multiple autoimmune and inflammatory conditions, identifies 30 miRNAs with specific immune functions (Tomankova et al., 2011)

Table 10. Multiple mechanism through which dysfunction of the immune system is associated with IBD

### 1.3.2 Dysbiosis

#### 1.3.2.1 Host / microbial homeostasis

There is a symbiotic relationship between man and his microflora that has been crucial throughout evolution. In return for a warm home and steady flow of food, microflora assists us through important metabolic, trophic and protective functions (Guarner and Malagelada, 2003, Tlaskalová-Hogenová et al., 2004). Disruption of this relationship is associated with dysfunction of the immune system either through loss of microbial immunological assistance, inappropriate activation or direct disruption of the host immune response (Sekirov et al., 2010,

Kaur et al., 2011). Imbalance or inappropriate response to microflora is considered a major contributor to the pathogenesis of IBD (Maloy and Powrie, 2011).

Microflora process about 20-60g of carbohydrate and 5-20g protein daily from substrates that would otherwise pass through and be wasted. Microflora also plays a key role in vitamin and micronutrient absorption as well as the production of short chain fatty acids (e.g. butyrate). Many studies have shown that short chain fatty acids have a protective effect against UC (Tonelli et al., 1995), possibly through actions which include increased epithelial cell proliferation (Alam et al., 1994) and differentiation (Gordon et al., 1997). A healthy balance of microflora also prevents proliferation of single organisms through competition for nutrition and other factors.

A feature of IBD is that microflora biodiversity appears restricted. Scanlan et al. (2006) compared the levels and stability of *Clostridium* spp, *bacteroides* spp, bifidobacteria and lactic acid bacteria in the faeces of healthy individuals and patients with Crohn's and showed that the floral community in Crohn's is more restricted and less stable over time, both of which were correlated, but not causal to, inflammatory activity. A stable and well balanced population of luminal flora is necessary for epithelial homeostasis.

Manipulation of the luminal microflora can be achieved through use of probiotics, antibiotics or faecal stream diversion. VSL#3, a probiotic, has achieved some favour as a safe option to treat active UC, pouchitis (Bibiloni et al., 2005, Pham et al., 2008) and prevent relapse of UC, although there is no clear evidence for its use in Crohn's. Ciprofloxacin and metronidazole are used in the treatment of pouchitis and fistulating perianal Crohn's (Sartor, 2004) and faecal stream diversion with an ileostomy prevents post-operative neoterminal ileum recurrence in Crohn's (Rutgeerts et al., 1991).

### 1.3.2.2 Is there a causative agent?

The microbiome between individuals varies greatly, and within an individual can fluctuate rapidly. Many factors contribute to floral composition including birthing method, weaning regime, diet, exposure to antibiotics and environmental factors.

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Recently host genotype has been shown to have a prevailing influence over environmental factors for luminal composition (Zoetendal et al., 2001). A healthy GI tract flora is composed of over 5000 different species of which up to 10% are aerobes. A small number of aerobes are potentially pathogenic, but are kept in check by mechanisms discussed earlier. In active Crohn's the balance is tipped towards a higher percentage of aerobic bacteria compared with quiescent IBD or active UC (Giaffer et al., 1991).

Many organisms have been implicated in the aetiology of IBD and experimental colitis although results have been too inconsistent to draw conclusions. Experimental models have shown some benefit from the eradication of individual pathogens (Videla et al., 1994), but this benefit has not proven to be clinically beneficial in IBD. Two specific exceptions are the use of ciprofloxacin and metronidazole for the treatment of perianal Crohn's and pouchitis, a phenomenon which may allude to distinct aetiological pathway in these 2 specific phenotypes.

Organisms implicated in the aetiology of IBD include; Salmonella, Shigella, Yersinia, Listeria monocytogenes (Subramanian et al., 2006), Escherichia coli, Bacteroides spp, Clostridium spp, Bifidobacteria spp, Mycobacteria spp, Campylobacter, Table 11.

Invasive bacteria are potentially of much greater significance. Patients with IBD have increased numbers of bacteria attached to their epithelial surfaces and an increased bacterial load in the intercellular and intracellular compartments compared with healthy individuals (Swidsinski et al., 2002). Although no single agent has been singled out, Table 11.

Despite experimental evidence of a strong link between the microbiome and inflammation, in practice a causal link remains elusive, and manipulation of the microbiome is only partially beneficial in a distinct group of patients. It is still likely however that the luminal flora plays a crucial role in the activation or perpetuation of IBD.

Pathogen	Technique used		Ref.
Mycobacteria spp	Biopsy wash culture	Not detected	1
Mycobacterium tuberculosis	RT-qPCR	Not detected in biopsies CD or UC	2
Mycobacterium avium	RT-qPCR	Not detected in biopsies CD or UC	2
Mycobacterium paratuberculosis	RT-qPCR	Not detected in biopsies CD or UC	2
	PCR (resection specimens)	MP present in 65.0% (CD) vs. 4.3% (UC) vs. 12.5% normal	3
Bacteroids spp	Biopsy wash culture	Main anaerobe in IBD and control, significantly higher count in CD and UC vs. control	1
	Biopsy RT-qPCR	Present in 16% UC, 30% CD, 11% non IBD inflammation, and 0% controls	1
Bacteroids vulgatus	RT-qPCR	100% (CD and UC) vs. 50% controls	2
	Stool culture	No difference CD vs. UC vs. control	4
Enterobacter spp	Biopsy wash culture	Main aerobe in IBD and control, significantly higher count in CD and UC vs. control	1
Escherichia coli	RT-qPCR	Biopsies 63% ( CD), 55% (UC) vs. 22% controls	2
	Biopsy RT-qPCR	Present in 21% UC, 20% CD, 27% non IBD inflammation and 5% controls	1
	Stool culture	Increase in total numbers in active CD vs. quiescent CD, UC and controls	4
Klebsiella	Stool culture	No difference CD vs. control	4
Proteus spp	Stool culture	No difference CD vs. control	4
Streptococcus viridans	Stool culture	No difference CD vs. control	4
Enterococcus faecalis	Stool culture	Decreased in active CD vs. quiescent CD, UC and controls	4
Diphtheroid bacilli	Stool culture	Increased in active CD vs. quiescent CD	4
Lactobacillus	Stool culture	Lower count in CD	4
Bifido bacteria	Stool culture	Lower in CD vs. UC and controls	4
Clostridium spp	Stool culture	No difference CD vs. UC vs. control	4
Listeria	Biopsy wash culture	Not detected	1
pseudomonas	Biopsy wash culture	Not detected	1

Table 11. A summary of infectious agents and their association with IBD. 1 (Swidsinski et al., 2002), 2 (Fujita et al., 2002), 3 (Sanderson et al., 1992), 4 (Giaffer et al., 1991).

### 1.3.3 Barrier dysfunction in IBD

Dysfunction of the barrier can be divided into 3 overlapping areas; a decreased mucus barrier, altered mucus constituents and a leaky epithelial cell barrier.

Both human and rodent models have shown that a decreased mucus barrier is associated with colitis. A decreased mucus barrier is seen in patients with inactive UC compared with healthy individuals (Cope et al., 1988), and induced deficiency of mucus in *Muc2<sup>-/-</sup>*, the major constituent of mucus, in rodent models increases the rate of spontaneous colitis (Velcich et al., 2002). Together this suggests that a reduced mucus barrier predisposes to IBD. Interestingly, helminth therapy has been shown to successfully treat otherwise resistant forms of UC by a process which stimulates mucus production through the increased expression of IL-22, and GM-CSF has also been shown to improve IBD by mechanisms which may include increasing DC and macrophage secretion of IL-23 which up-regulates IL-22.

Immunoglobulins and antimicrobial peptides are important constituents of intestinal mucus which prevent microbes from invasion. In healthy individuals the intestinal mucus is rich in IgA and antimicrobial peptides such as the defensins, lysozymes and cathelicidins. In mucosal washings from patients with IBD the concentration of IgG which can trigger inflammatory cascades, is 200-300 times higher compared with controls (Macpherson et al., 1996). Enhanced bacterial survival and invasion is encouraged by decreased mucosal defensin concentration. Reduced concentration of  $\alpha$ -defensin is seen in the mucosa of patients with Crohn's, and is particularly marked in Ileal Crohn's associated with mutations of NOD2 (Wehkamp et al., 2004) and defective Paneth cell function (Lala et al., 2003).

There is growing evidence that there is a strong association between increased epithelial barrier permeability and IBD (Clayburgh et al., 2004, Laukoetter et al., 2008). Older studies report an increased permeability to normally unabsorbable carbohydrates in active UC (Nejdfors et al., 1998) and Crohn's (Jenkins et al., 1987) but draw their conclusions from a comparison with healthy individuals, making the inference that the increased permeability is due to the IBD diagnosis rather than the increased permeability normally seen in an inflamed epithelium. More recent observational studies however have shown an increased epithelial

permeability in a specific subset of high risk individuals who have not yet been diagnosed with Crohn's (May et al., 1993) and 20% of patients prior to a flare (Wyatt et al., 1993). Both populations had no signs of active inflammation suggesting a causal relationship to IBD. On a molecular level, both active and inactive IBD have a differential expression of junctional proteins compared with healthy colon and that these proteins can be disrupted in vitro by the inflammatory cytokines commonly increased in IBD; TNF- $\alpha$ , INF- $\gamma$  and IL-13 (Prasad et al., 2005). Also, in clinical practice, a feature of successful rescue therapy with TNF- $\alpha$  inhibitors is the decreased epithelial permeability, although this appears to be through a reduction in epithelial cell apoptosis rather than an influence on junctional proteins (Zeissig et al., 2004).

### 1.3.4 Dysfunction of the innate immune system in IBD

Inflammatory Bowel Disease has long been thought to be a disorder of T cell function, however the genetic association between specific Crohn's phenotypes and the NOD2/CARD15 gene has brought abnormalities of the innate immune system to the fore.

#### 1.3.4.1 NOD2/CARD15 mutations

NOD2 is an intracellular PRR that is constitutively expressed in macrophages, neutrophils and dendritic cells (Ogura et al., 2001b), as well as Paneth and epithelial cells (Ogura et al., 2003). NOD2 is essential for NF- $\kappa$ B activation and down-stream inflammatory cytokines production by antigens which contain a muramyl dipeptide (MDP) moiety. The consequences of loss of function of NOD2 are seen in the genetically inherited Blau syndrome which is characterised by multisystem granulomata. 30% of patients with Blau syndrome suffer a granulomatous colitis akin to Crohn's.

Recognition of NOD2 mutations in Crohn's were first reported in 2001 by Ogura et.al. (2001a) and are seen in between 20% and 40% of patients with Crohn's, these patients tend to present at an earlier age, follow a different disease activity phenotype and require high rates of surgery (Alvarez-Lobos et al., 2005).

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Three specific NOD2 mutations; Arg702Trp, Gly908Arg and Leu1007fsinsC account for 82% of those seen in Crohn's in Western populations. 90% of the mutations are located in the LRR domain of the NLR and confer a 2-4 fold risk of developing Crohn's in heterozygotes and 20-40 fold risk in homozygote carriers (Cuthbert et al., 2002). The 3 mutations are however commonly seen in the healthy population with frequencies of 4.3%, 1.2% and 2.3% respectively (Hugot et al., 2007), this indicates that genetic penetrance is low and although associated with IBD, causation is through an interaction with other unknown risk factors.

Loss of function mutations of NOD2 can lead to; decreased defensins expression, decreased antigen recognition, decreased TCR binding by APCs through decreased CD80 and CD86 expression, reduced cytokine expression by leukocytes and decreased neutrophil migration and function (Yamamoto-Furusho and Korzenik, 2006) as well as a diminished response of macrophages to invasion through decreased IL-8 and neutrophil recruitment (Marks and Segal, 2008) and decreased DC activity with reduced autophagy, bacterial killing and antigen presentation (Cooney et al., 2010). All of these dysfunctions lead to a relative immunodeficiency disorder which allows enhanced bacterial survival and invasion.

### 1.3.4.2 Monocyte dysfunction

Dendritic cells play a crucial role in epithelial homeostasis, tolerance to luminal flora, immune resistance to potentially harmful luminal constituents and T cell development and function.

Dendritic cells are influenced by epithelial cells which secrete numerous factors to control their function. When exposed to EC derived TGF- $\beta$  and retinoic acid, DCs confer a suppressive role and promote the differentiation of naïve T cells into the FoxP3 T<sub>reg</sub> lineage (Iliev et al., 2009). Epithelial cells also express other regulatory factors such as TSLP which, through influence on DCs, promote barrier homeostasis and a T<sub>H</sub>2 favoured inflammatory response.

Dysfunctional macrophages have also been implicated with IBD through two broad mechanisms; firstly through abnormal bacterial handling a disorder linked

prominently with loss of function mutations in NOD2, and secondly through increased number and / or function of pro-inflammatory macrophages.

#### 1.3.4.3 Therapies for the innate immune response

The contribution of the innate immune system to the pathogenesis of IBD is reflected through the therapeutic pathways of several established and more novel treatments. Inhibitors of TNF- $\alpha$  and corticosteroids affect macrophages through increased apoptosis of pro-inflammatory macrophages and decreased macrophage pro-inflammatory cytokine release respectively. Macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) are growth factors that enhance important functions in the epithelial and phagocytic components of the innate immune system. Initial studies of recombinant GM-CSF (Sargramostim) in moderate to severe Crohn's achieved a remission rate of 53% at 8 weeks (Dieckgraefe and Korzenik, 2002) and although a larger randomised placebo controlled trial failed to achieve the earlier promising results it did show a significant improvement in CDAI score and quality of life measurement (Korzenik et al., 2005). Together these observations not only suggest a direct influence of the innate immune system in the pathogenesis of IBD but also that therapies aimed at improving the innate immune response have exciting potential.

#### 1.3.5 Dysfunction of the adaptive immune system in IBD

The hallmark of IBD is a polarised T cell response driven by abnormal antigen processing by APCs. The T cell response in IBD has 2 separate characteristics, the first is an increase in the ratio of pro-inflammatory to suppressive T cells subsets in the peripheral circulation as well as the intestinal mucosa (Eastaff-Leung et al., 2009). The second characteristic is abnormal behaviour of those lymphocytes resulting in hyper-reactive pro-inflammatory T cells or hypo-reactive suppressive T cells (Strober et al., 2007).

Mucosal lymphocytes were first described in 1847 (cited by Dobbins, 1986 p972) and have been studied closely in IBD for over 40 years (Selby et al., 1981, Selby et al., 1984, Caballero et al., 1995). Over recent years numerous distinct T cell subsets have been identified, many of which have abnormalities of number or function

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associated with IBD. The T cells which have been most prominently linked to the aetiology of IBD include the CD4<sup>+</sup> helper T cells; T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17 and T<sub>regs</sub>. Other lymphocyte subsets also increasingly implicated are the NK cells, CD8<sup>+</sup>, B cells and CD5<sup>+</sup> cells.

The T cell population is increased in active IBD by 2-3 fold compared with the mucosa of healthy individuals, although the balance of CD4<sup>+</sup> to CD8<sup>+</sup> is unaltered. In both normal mucosa and that of inflamed IBD, CD4<sup>+</sup> T cells predominate in the lamina propria, and CD8<sup>+</sup> cytotoxic cells predominate in the intraepithelial layer (Selby et al., 1984, Caballero et al., 1995).

The increased T cell population seen in IBD is a result of either sequestration of lymphocytes from the periphery under the control of chemotactic factors or increased central or peripheral differentiation of effector cells from naïve T cells, these issues along with functional changes in the cells of the adaptive immune system are discussed separately for the individual T cell subsets.

### 1.3.5.1 Regulatory T cell

Regulatory T cells play a crucial role in the maintenance of immune homeostasis. In mouse models they have been shown to prevent or reverse inducible colitis and in humans an imbalance of T<sub>reg</sub> numbers and/or function is associated with a number of autoimmune conditions, transplant rejection, inability to develop tolerance and the exaggerated pro-inflammatory response characteristic for IBD.

In the actively inflamed mucosa of individuals with IBD the number of T<sub>regs</sub> is increased, coinciding with a decrease in the number of T<sub>regs</sub> in the peripheral circulation (Maul et al., 2005, Eastaff-Leung et al., 2009, Holmen et al., 2006). In non-IBD colitis the increase in mucosal T<sub>regs</sub> is even greater and coincides with an increase in the number of peripheral T<sub>regs</sub> (Maul et al., 2005). In non-inflamed mucosa of both IBD patients and healthy individuals there are only a small numbers of T<sub>regs</sub>, but in areas of inflammation in the same patients there are increased numbers of T<sub>regs</sub>. Taken together, these results suggest that not only is the T<sub>reg</sub> pool in IBD smaller than in healthy individuals, but there may be dysfunctional trafficking to areas of inflammation. Potential abnormalities include

dysfunction of production, decreased  $T_{reg}$  stability and survival or de-differentiation into other T cell phenotypes.

$T_{regs}$  are produced centrally in the thymus or differentiated peripherally from naïve T cell precursors ( $T_H0$  cells). Under the influence of TGF- $\beta$  and IL-6,  $T_H0$  cells differentiate into either  $T_{regs}$  or  $T_H17$  cells establishing a reciprocal balance between inflammation and regulation (Siddiqui and Powrie, 2008, Kimura and Kishimoto, 2010). Under normal conditions the potentially deleterious effect of luminal substances or microflora is suppressed through the enhanced differentiation and function of  $T_{regs}$  by the secretion of TGF- $\beta$  by dendritic cells (Cai et al., 2010) and / or other factors. In the presence of STAT3 mediated signals, IL-6 (or IL-23)  $T_H0$  cells differentiate into  $T_H17$  cells at the expense of  $T_{regs}$ , allowing the inflammatory response to override  $T_{reg}$  suppression and promote host defence. Imbalance of  $T_{regs} / T_H17$  is a feature of IBD although the exact mechanism of this is unknown (Kimura and Kishimoto, 2010) but may include abnormalities within pathways involving IL-6, -21, and IL-23 amongst others (Eastaff-Leung et al., 2009). However, central to the differentiation, stability, survival and function of  $T_{regs}$  is the transcription factor FoxP3, through which the factors mentioned above function.

FoxP3 is encoded by the FOXP3 gene, in humans, loss of function mutations result in the IPEX (Immunodysregulation, Polyendocrinopathy, Enteropathy X-linked) syndrome, and in mice deficiency results in multisystem inflammation and autoimmunity and a susceptibility to colitis. FOXP3 mutations are not known in IBD although pathways involving the FoxP3 transcription factor and downstream pathways are central to the idea of  $T_{reg}$  dysfunction in IBD.

Increased expression of FoxP3 results in increased number and activity of  $T_{regs}$ , it classically occurs through induced phosphorylation of the SMAD complex and STAT5 as a result of TGF- $\beta$  and IL-2. Recently the differential expression of 5 miRNAs have been demonstrated in  $T_{regs}$ , two of which, miR-21 and miR-31 are associated with abnormal FOXP3 expression (Rouas et al., 2009) and are differentially expressed in the mucosa of IBD (Fasseu et al., 2010). FOXP3 expression drives approximately 13 different mechanisms through which  $T_{regs}$  exert their suppressive function; TGF- $\beta$ , IL-2, CTLA4, IL-10 and IL-35 expression in

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humans, and in-vitro experiments; Granzyme B, Perforin, IFN- $\gamma$ , IL-9, HO-1, cAMP, Galectins and adenosine (Tang and Bluestone, 2008).

Continued stability and function of  $T_{reg}$  requires TGF- $\beta$ , IL-2 and miRNAs (discussed later). The lack of TGF- $\beta$  and IL-2 stimulation is demonstrated in TGF- $\beta$ <sup>-/-</sup> and IL-2<sup>-/-</sup> knockdown mice which succumb to fatal inflammation in much the same way as FOXP3<sup>-/-</sup> mice do, transfer of  $T_{reg}$  abrogates this. Lack of on-going stimulation reduces  $T_{reg}$  numbers by either death or dedifferentiation. In adoptive transfer models about 10-15% of  $T_{reg}$  lose their expression of FOXP3 resulting in a reduced expression of CD25, CTLA-4 and GITR and increased IL-2.

Decreased production of FoxP3 occurs as a result of IL-6, IL-4, and strong TCR signals through STAT6, GATA3, and mTOR, respectively, continued loss of FoxP3 production can lead to a change of phenotype from  $T_{reg}$  to  $T_H17$  cells with an increased expression of IL-2 and IFN $\gamma$ , a process known as plasticity (Zhou et al., 2009).

### 1.3.5.2 $T_H1$ / $T_H2$

Precursor  $T_H0$  lymphocytes differentiate into  $T_H1$ ,  $T_H2$  or other cell types depending on the cytokine milieu; see section 1.2.4.3.1 and Figure 7. Traditional theories describe Crohn's as a  $T_H1$  mediated inflammatory condition with an increase in cytokines IL-12, IFN- $\gamma$  and TNF- $\alpha$ , and UC as a  $T_H2$  mediated inflammatory condition with an increase in cytokines IL-4 and IL-13. However rather than being mutually exclusive to either UC or Crohn's,  $T_H1$  and  $T_H2$  responses are exaggerated in both diseases along with a complex interplay of other inflammatory and/or cytokine responses associated with other cell types.

### 1.3.5.3 $T_H17$ cells

There is an established relationship between  $T_H17$  cells, the pro-inflammatory cytokine IL-17 and IBD (Nielsen et al., 2003). Total numbers of  $T_H17$  cells are increased in the peripheral blood of individuals with inactive IBD (Eastaff-Leung et al., 2009), and IL-17 expression is enhanced in the intestinal mucosa and peripheral blood lymphocytes of Crohn's (Fina et al., 2008) and mucosal CD4<sup>+</sup> T cells in resected UC (Kobayashi et al., 2008).

What drives the increased differentiation and / or effector function of T<sub>H</sub>17 cells in IBD is largely unknown. Experimental models have shown opposing roles for bacteria between the small and large intestine; in the small intestine bacteria appear necessary for IL-17 production (Ivanov et al., 2008) through mechanisms which include APC derived IL-6, IL-23 and TGF- $\beta$  (Atarashi et al., 2008), compared with the large intestine in which the absence of bacteria is associated with a higher level of IL-17 due to the loss of bacteria derived IL-25 (IL-17E) which would normally counter-regulate IL-23 produced by lamina propria macrophages (Zaph et al., 2008).

The genetic link to dysfunction of the T<sub>H</sub>17 cell lineage is underpinned by the recent discovery of many genetic susceptibility loci which are involved in IL23 / T<sub>H</sub>17 signalling, including; IL23R, IL12B, JAK2, TYK2, STAT3, IL10, IL1R2, REL, CARD9, NKX2.3, ICOSLG, PRDM1, SMAD3 and ORMDL3 (Lees et al., 2011). The interplay between these loci and T<sub>H</sub>17 / IL-17 imbalance has yet to be determined.

### 1.3.6 Loss of tolerance

Tolerance is the mechanism that prevents the immune system triggering an inflammatory response to antigens that are not harmful. There are 2 aspects of tolerance; the first is tolerance to self-antigens, the second is tolerance to the commensal luminal flora.

Tolerance to self-antigens is created centrally in the thymus and bone marrow during lymphocyte development, and/or peripherally through suppression of active self-reactive lymphocytes. Breakdown of these mechanisms is a characteristic of autoimmune diseases, and although not a recognised feature IBD there is an aspect of autoimmunity that may play a part in IBD. "*Molecular mimicry*" is a process in which an antigen creates an appropriate response and is cleared, but due to the similarity between it and normally innocuous luminal contents or self-antigens an autoimmune response can ensue and perpetuate the initial inflammation (Oldstone, 1998).

Continuous exposure to commensal flora is essential for the maintenance of epithelial homeostasis and protection against injury (Rakoff-Nahoum et al., 2004).

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Exposure and the development of tolerance to commensal non harmful bacteria is achieved through luminal sampling and bacterial translocation, both of which are normal processes and result in bacteria or components of bacteria being present in the lamina propria (Swidsinski et al., 2002) and regional lymph nodes of healthy individuals.

A feature of IBD is the loss of tolerance to commensal flora. Many different mouse models that are rendered susceptible to colitis through gene knockout have shown that commensal bacteria are required to induce inflammation; growth under germ free conditions abrogates this. In humans, inflammation can be likewise avoided by removing commensal bacteria by faecal stream diversion (Harper et al., 1985). The adaptive immune system in IBD demonstrates increased numbers of activated T cells against luminal antigens, and increased levels of IgG secretion in the mucus and mucosal epithelium. Together this shows that the presence or abnormal processing of commensal bacteria is required to trigger inflammation and that the immune system in IBD is hyper-responsive to commensal, normally harmless, bacteria. Abnormal bacterial processing by mutations in NOD2/CARD15 gene have also been suggested mechanisms (Guarner and Malagelada, 2003).

### 1.3.7 Other aetiological associations with IBD and their effects on the immune system

The following broad areas have all been implicated in the aetiology of IBD.

#### 1.3.7.1 Influence of genetics

In recent years sub-populations based on genetic or immunological phenotypes have emerged which, show marked differences in disease outcome (Targan and Karp, 2005). The most obvious indication of the association of genetics and IBD is the increased incidence of IBD within families and racial groups. There is a high concordance rate between monozygotic twins, for UC it is 10-15% and for Crohn's 30-35%. Certain racial groups have a higher prevalence rate of IBD including northern Europeans and Ashkenazi Jews.

Early HLA linkage studies suggest an increased risk of UC and Crohn's for people who carry the HLA-DR2 and HLA-DR1 subtypes respectively, although these links are inconsistent (Naom et al., 1996). In 2001, a NOD2 frame shift mutation was first reported as a risk factor for Crohn's (Ogura et al., 2001a). By December 2011 a total of 99 susceptibility loci have now been identified for IBD, 71 for Crohn's (Franke et al., 2010) and 47 for UC (Anderson et al., 2011), 30 of which are present in both forms of IBD (Lees et al., 2011).

Between 20% and 40% of Crohn's have variants in the NOD2 gene and those with NOD2 mutations are more likely to present at an earlier age, have a more aggressive disease phenotype, 3.6 times more likely to have stricturing disease, and require surgery (Alvarez-Lobos et al., 2005). Other prominent gene associations with Crohn's include those that influence bacterial handling; ATG16L1 and IRGM, both involved with autophagy. In ulcerative colitis the IL12B gene (T<sub>H</sub>17 cell function) is prominent.

The influence of genetic variants is stronger for Crohn's than UC, although the incomplete penetrance suggests other factors play a role. There is also an important difference between racial groups with IBD and genetic variants, for example the NOD2 mutation is less commonly seen in the Asian population with Crohn's compared with whites.

### 1.3.7.2 Environment influences

Inflammatory Bowel Disease was a rare diagnosis in Asia until the 1980s, but the incidence is climbing (Ahuja and Tandon, 2010), and although the point prevalence remains well below that of western countries, the total numbers of people with IBD is rapidly increasing. The general consensus is that this increase is due to a western lifestyle and changing environmental influences (Mowat et al., 2011).

This pattern of increased incidence is also seen within Europe in people who have emigrated from less developed areas (Barreiro-de Acosta et al., 2011). Taken together the increasing rates within populations previously thought to have a low incidence and these same populations that emigrate to more developed areas

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suggest that environmental factors related to industrialisation play an important role in the aetiology of IBD, particularly UC (Barreiro-de Acosta et al., 2011).

One suspected environmental factor is the lack of exposure to environmental pathogens immediately after birth and in childhood. During development, the immune system is influenced by orally consumed environmental pathogens, lack of exposure and the resulting decreased oral tolerance is the key idea underpinning the “Hygiene hypothesis” (Strachan, 1989).

Other environmental and childhood factors which alter immune development and influence the risk of subsequently developing IBD include breast feeding, tonsillectomy, appendectomy, immunisation and common childhood infections such as measles and chicken pox (Hansen et al., 2011). Other environmental influences include tobacco (Silverstein et al., 1989), pollution, diet, oral contraceptive pill and non-steroidal anti-inflammatory drugs (Hansen et al., 2011).

Cigarette smoke is of particular interest due to the strong protective effect of cigarette smoke in UC and the opposing increased association with Crohn's. The exact mechanism has not been defined but in their review McGilligan et al. (2007), suggest several components of cigarette smoke interfere with various aspects of the immune system including effects on epithelial integrity and the  $T_H1/T_H2$  balance.

## 1.4 MicroRNA

### 1.4.1 Before microRNA

Although a modern phenomenon the story of MicroRNA (miRNA) can be traced back over 50 years to the work of Jaques Monad, Andre Lwoff and Francis Jacob who were awarded the 1965 Nobel Prize for Medicine “*for discoveries into the genetic control of enzyme and virus synthesis*”. Their work involved the synthesis of a simple sugar (lactose) in bacterium and its control by a gene repressor which they believed to be RNA; unfortunately they were only ever able to isolate proteins.

A quarter of a century later, Professors Altman and Čech received the Nobel Prize for Chemistry in 1989 for their independent contributions to the discovery of the catalytic properties of RNA<sup>4</sup>. Prior to their discoveries in the early 1980’s only proteins were thought to have catalytic properties and the sole role of RNA was to act as a messenger molecule between the genetic code stored in DNA and the cellular mechanism responsible for protein synthesis.

The discovery that RNA has properties other than those of a messenger has two important implications; the first is an explanation of how genetic code can be deciphered and proteins synthesised in the absence of enzymes, a fundamental question in evolution, the second paved the way to work on RNA as a regulator of gene expression.

In the late 1980s it was noticed that the Lin-4 gene controls the progress of *C. Elegans* from the first larval stage to the second, but what was interesting is that Lin-4 does not code for a protein, it codes for a pair of short length RNAs (Ambros, 1989). It was also noticed that the Lin-4 RNAs had antisense complementarity to multiple sites in the 3’ UTR of the Lin-14 gene, and that increased lin-4 expression reduced the amount of Lin-14 protein without reducing the level of Lin-14 mRNA. Together this shows that Lin-4 RNAs pair to the Lin-14 3’UTR to specify translational repression of the Lin-14 pathway (Lee et al., 1993, Wightman et al., 1993).

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<sup>4</sup> The Nobel Prize in Chemistry 1989 presentation speech given by Professor Bertil Andersson gives a succinct account of the background to the work of both Altman and Čech and the origins of RNA as enzymes. Available at <http://nobelprize.org> (accessed 10th Oct 2011).

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The next two breakthroughs came in 2000 with the identification of a second gene, let-7 (Reinhart et al., 2000). Let-7 is again involved in the larval development of *C. Elegans*, but the important discovery is that it is conserved across species, including humans (Pasquinelli et al., 2000). Within one year another 30 short length RNAs were identified in humans and this abundant class of short length RNA was given the name miRNA (Lagos-Quintana et al., 2001, Lau et al., 2001, Lee and Ambros, 2001).

### 1.4.2 MicroRNA

MicroRNAs are a family of single-stranded and short-length RNA (19-23nt) that exert their effect on gene regulation and cell phenotype by preventing the translation of mRNAs into protein (Bartel, 2004). To date 1600 human precursor, and 2042 mature miRNA sequences have been registered on miRBase release 19 (Aug, 2012), these follow nomenclature rules outlined Table 12.

Example miRNA = has-miR-146a-5p	
hsa	Tells us it is human, homo sapien
miR	Capitalisation of the "R" infers that the miRNA is the mature sequence, opposed to "r" which refers to the miRNA precursor, the genomic locus, the primary transcript, or the extended hairpin that includes the precursor.
29	Named sequentially, this miRNA belongs to the 29 family. The same number is used for miRNA of different species that are orthologous, but conveys no information about functionality.
b	Paralogous miRNAs that differ in 1 or 2 nt.
-5p/-3p	the same mature miR originate from opposite arms of the same pre-miRNA

Table 12 Mature miRNA terminology, used by miRBase. There are 5 sections to the nomenclature (Ambros et al., 2003).

A canonical schematic of miRNA biogenesis is shown in Figure 8, although each step can be achieved through alternative pathways (Winter et al., 2009, Bartel, 2004). The majority of miRNA genes are intergenic and transcribed from the DNA template independently by RNA polymerase 2. However between 20% and 40% of human miRNA genes originate in the introns of larger protein and non-protein

genes or the exons of long protein coding transcripts, and are therefore transcribed and regulated along with their host genes. It follows that the highly conserved relationship between miRNA and host mRNA is due to the necessity for a coordinated expression, although no example exists in humans, mir-7 is a striking example of this phenomenon in insects and lower order mammals (Aravin et al., 2003).

MiRNAs are usually transcribed by RNA polymerase 2 which binds to a promoter region within the DNA sequence found near the hairpin loop of the pre-miRNA. The stem loop sequence forms part of a long polycistronic miRNA precursor, pri-miRNA, which may contain up to 6 miRNA precursors. Alternatively RNA polymerase 3 transcribes certain miRNA, especially those with Alu sequences.

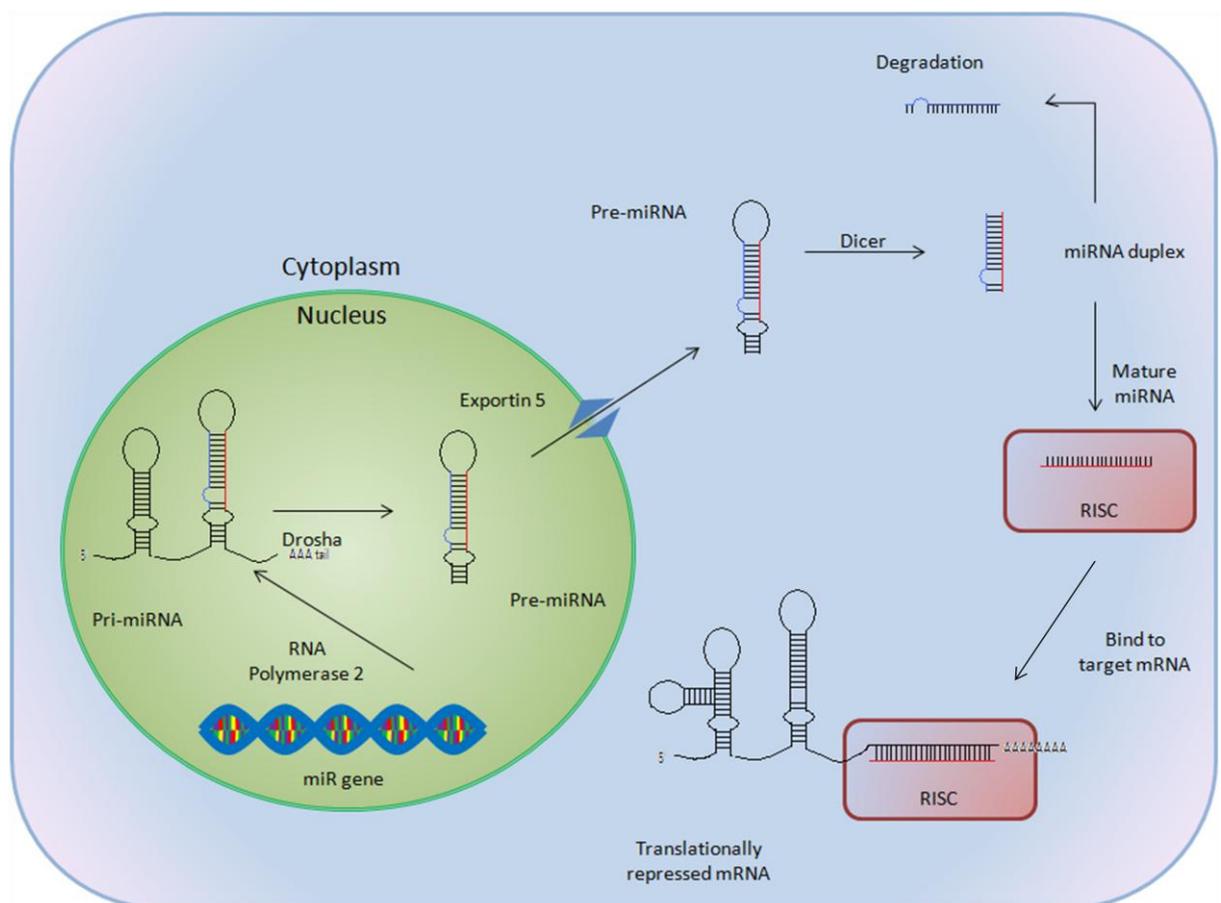


Figure 8. A schematic of miRNA biogenesis

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The pri-miRNA is recognized by a nuclear protein known as DiGeorge Syndrome Critical Region 8 (DGCR8) which associated with the enzyme Drosha. Together the Drosha / DGCR8 complex utilises the catalytic component of Drosha, RNase 3, to liberate the 70-80nt hairpin structure termed pre-miRNA (precursor-miRNA). Importantly, RNase 3 leaves a two-nucleotide overhang at its 3' end which is required for nuclear export to the cytoplasm. Alternatively pre-miRNAs are spliced directly from introns, bypassing the Drosha / DGCR8 complex, these products are known as "Mirtrons" and have recently been found to exist in mammals (Berezikov et al., 2007).

Pre-miRNAs are exported from the nucleus in a process involving the nucleocytoplasmic shuttle Exportin-5, which recognises the two-nucleotide overhang left by RNase 3 processing.

In the cellular cytoplasm the pre-miRNA hairpin is cleaved by the RNase III enzyme Dicer. Dicer recognises the 3' end of the hairpin and cuts away the loop resulting in a miRNA:miRNA duplex about 22nt in length. Only one strand is usually then incorporated into the RNA-induced silencing complex (RISC) based on its thermodynamic stability and strength of base pairing relative to the other strand, although either end could potentially act as a functional miRNA

RISC is the functional component of the process, and contains multiple independent elements including members of the Argonaute (Ago) family, TRBP (transactivating response RNA binding protein), PACT (protein activator of the interferon induced protein kinase), the SMN complex, fragile X mental retardation protein (FMRP), and Tudor staphylococcal nuclease-domain-containing protein (Tudor-SN).

Argonautes are the most important components of RISC. Argonautes bind the mature miRNA through two RNA binding domains: a PAZ domain that can bind the single stranded 3' end of the mature miRNA and a PIWI domain which interacts with the 5' end of mature miRNA. Binding orientates the miRNA for interaction with the target mRNA

Gene silencing occurs through mRNA degradation or by preventing mRNA from being translated.

The association between miRNA and the immune system began in 2004 when Chen et al observed the selective expression of miR-142a, miR-181a and miR-223 in immune cells (Chen et al., 2004). Since this initial observation, miRNAs have been associated with the maturation, proliferation, differentiation and activation of immune cells (Cobb et al., 2005, Cobb et al., 2006). At least 28 miRNAs have been established as integral to the proper function of many aspects of the immune system including; B cell development (miR-150 and miR-17), granulopoiesis (miR-223), immune function generally (miR-155 and miR-146) and B cell lymphoproliferative disorders (miR-155 and miR-17), reviewed by Tili et al. (2008), O'Connell et al. (2010) and Garzon and Croce (2008). Many more have been associated with the aetiology of numerous autoimmune and inflammatory conditions.

### 1.4.3 MicroRNA in IBD

MicroRNAs are essential for the normal function of inflammatory cells and immune homeostasis. Interruption of miRNA biogenesis, using either Drosha or Dicer knockout mice promotes Inflammation of the colon, decreased regulatory T cell function and rapidly fatal autoimmunity (Chong et al., 2008, Cobb et al., 2006).

There is growing evidence that specific miRNAs play a crucial role in immune regulation and the aetiology of IBD. There are 4 prominent miRNA expression profiles reported in the literature for UC and Crohn's (Wu et al., 2008, Wu et al., 2010, Fasseu et al., 2010, Van der Goten et al., 2012). The first group to publish a miRNA profile for UC was Wu et al. (2008) who studied the expression of 553 miRNAs by array and showed the altered expression of 18 miRNAs, of which 11 were confirmed with qPCR. More recently Fasseu et al. (2010) have used TaqMan™ MicroRNA Array (Applied Biosystems) to study 321 miRNAs and Van der Goten et al. (2012) used the Affymetrix GeneChip® miRNA 2.0 array to study 1105 miRNAs. The pooled results show that 72 miRNAs have either an increased or decreased expression in IBD, although only 14 miRNA are reported as differentially expressed by 2 or more of the 4 studies, Table 13 and Table 14.

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Reference	Tissue	phenotype	increased	decreased	comment
Wu et al. (2008)	Mucosal biopsies	Active disease	miR-16 miR-21 miR-23a miR-24 miR-29a miR-126 miR-195 Let-7f	miR-192 miR-375 miR-422b	
Fasseu et al. (2010)	Mucosal biopsies	Active disease	miR-7 miR-31 miR-135b miR-223		5/8 (63%) patients on a disease modifying agent. Phenotype excluded total colitis. Array cut of is fold difference >5 or <0.2
		Quiescent mucosa in patient with active UC	miR-196a		Histological grade 1 included as non-inflamed, therefore some signs of inflammation present microscopically
		Active and quiescent mucosa	miR-29a miR-29b miR-126 miR-127-3p miR-324-3p	miR-188-5p miR-215 miR-320a miR-346	Those miRNA which show a differential expression in both active and non-active inflammation in the same colon at the same time
		Quiescent UC	miR-29a miR-29b miR-30c miR-126 miR-127-3p miR-196a miR-324-3p	miR-188-5p miR-199a-3p miR-199b-5p miR-215 miR-320a miR-346 miR-370	
Van der Goten et al. (2012)	Mucosal colon biopsies	Active disease	let-7i-5p miR-21-3p miR-21-5p miR-27a-5p miR-29b-1-5p miR-29b-2-5p miR-31-5p miR-99b-3p miR-138-5p miR-146a-5p miR-146b-3p miR-146b-5p miR-150-5p miR-155-5p miR-193-5p miR-212-3p miR-223-3p miR-363-3p miR-503 miR-551b-5p miR-584-5p miR-650 miR-665 miR-708-5p miR-1273d miR-1972	miR-10b-3p miR-10b-5p miR-141-3p miR-147b miR-192-3p miR-192-5p miR-194-3p miR-194-5p miR-196a-5p miR-196b-3p miR-196b-5p miR-200a-3p miR-200a-5p miR-200b-3p miR-200b-5p miR-215 miR-3201 miR-335-5p miR-375 miR-378-3p miR-378-5p miR-378c miR-422a miR-4284 miR-429 miR-1973	Undefined patient group

Table 13. miRNAs that are differentially expressed in UC. Three studies which use differing microarray techniques to discover which miRNAs are differentially expressed in the mucosa in UC.

Reference	Tissue	phenotype	increased	decreased	comment
Wu et al., (2010)	Mucosal biopsies	Active colonic CD	miR-23b miR-106 miR-191	miR-19b miR-629	
		Terminal ileal CD	miR-16 miR-21 miR-223 miR-594		
		Colon region specific differential expression	miR-31 (TI) miR-215 (TI) miR-22 (TI) miR-422b (caecum) miR-23a (sigmoid) miR-126 (transverse colon and rectum) miR-7d (TI and sigmoid)	miR-19b (TI) miR-26a (caecum and transverse colon) miR-320 (TI and rectum)	
Fasseu et al., (2010)	Mucosal biopsies	Active disease	miR-9 miR-126 miR-130a miR-181c miR-375		Inclusion criteria following the array, RQ >5 or <0.2, all p<0.05
		Non inflamed	miR-9 miR-30a miR-30c miR-223		
		Active and non inflamed	miR-26a miR-29b miR-30b miR-34c-5p miR-126 miR-127-3p miR-133b miR-155 miR-196a miR-324-3p miR-21 miR-22 miR-29c miR-31 miR-106a miR-146a miR-146-5p miR-158		

Table 14. miRNAs that are differentially expressed in Crohn's disease. Two studies which use differing microarray techniques to discover which miRNAs are differentially expressed in the mucosa in UC.

The poor concordance between the studies is likely to represent sample heterogeneity and differing study design. Interpreting and applying the results are even more challenging due to the poorly described patient phenotype and group

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heterogeneity. Characteristics which are combined into the same group include; treatment differences as diverse as not treated versus steroid or biological therapy, phenotype differences such as distal versus extensive disease, sample differences taken from urgent colectomy versus surveillance biopsies or tissue from the distal versus the proximal colon. To be clinically relevant the patient characteristics must be homogeneous and grouped into clinically relevant and distinguishable groups.

Studies into the impact of differentially expressed miRNAs in IBD are in their infancy, although their influence on several mechanisms has been suggested including; crosstalk between epithelial cells and the innate immune system (Biton et al., 2011) and influence on the differentiation of  $T_H1$  lymphocytes and the adaptive immune system (Tili et al., 2007).

The limited work on IBD is in contrast to the vast number of studies which have implicated miRNAs in the development of colitis in experimental models. Although induced colitis is an entirely different condition to IBD, interpreted correctly, the ideas and themes provided by experimental models provide important clues regarding the causes of IBD in humans.

MiR-155 in particular has been implicated in numerous biochemical pathways that regulate the immune response. In the innate immune system, miR-155 is expressed by macrophages in response to stimulation by LPS, IFN- $\gamma$ , TNF- $\alpha$  and IL-1 $\beta$ . (Ceppi et al., 2009) In dendritic cells, miR-155 controls the intensity of the inflammatory response by down regulating IL-1 $\beta$  and IL-6. In the adaptive immune system miR-155 shows greatly increased expression in activated B and T cells. (Rodriguez et al., 2007) In miR-155 null mice, CD4<sup>+</sup> cells are biased towards  $T_H2$  differentiation (Rodriguez et al., 2007) and the production of  $T_{regs}$  are greatly reduced (Kohlhaas et al., 2009) through the targeting of SOCS1.

Many miRNAs have been shown to be involved in lymphocyte function in animal models (Ceppi et al., 2009, Tsitsiou and Lindsay, 2009), although extrapolation into human disease must take into account the poor concordance between mouse and human lymphocytes, the differing miRNA signature between lymphocyte subsets

(Rossi et al., 2011) and the uncertain functional concordance between miRNAs that are given the same numerical name across species.

A T cell subset of particular interest in IBD is the regulatory T cell. Dysfunction is associated with experimental and human colitis. Numerous miRNAs have been shown to be differentially expressed in human T<sub>regs</sub>, Table 15, and shown to be important for T<sub>reg</sub> stability (Zhou et al., 2009).

	Differentially expressed miRNA	Tissue type	reference
T <sub>reg</sub> (CD4 <sup>+</sup> CD25 <sup>+</sup> )	Increased	Human Umbilical cord blood using TaqMan <sup>®</sup> low density Array	Rouas et al (2009)
	Decreased		

Table 15. Differentially expressed miRNA in human T<sub>regs</sub>

## 1.5 Aims and Objectives

It is clear that in health miRNA play an important role in the regulation of many inflammatory pathways. Loss of regulation through the abnormal expression of certain miRNAs is associated with many inflammatory processes. It follows that the initiation and / or perpetuation of IBD is likely to fall under the influence of a number of abnormally expressed miRNAs.

The aim of this thesis is to demonstrate that there is an altered expression of a distinct group of miRNAs in the intestinal mucosa of patients with active UC, and to demonstrate that the altered expression of these miRNAs influences the function of immune cells leading to a pro-inflammatory state which predisposes to or propagates IBD.

The objectives are:

1. To show that there is the abnormal expression of certain miRNAs in the intestinal mucosa of patients with active sigmoid UC.
2. To demonstrate the abnormal expression of miRNAs in mucosal lymphocytes and mucosal CD4<sup>+</sup> lymphocytes in patients with UC.
3. To identify a miRNA target.
4. To demonstrate that miRNA expression influences the expression of the target gene product.

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## **Chapter 2. Materials and Methods**

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## 2.1 Equipment and materials

### 2.1.1 Capital equipment

7900HT Fast Real-Time PCR System (Applied Biosystems™, USA)

MagNA Lyser (Cat # 03358968001, 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)

Used to centrifuge 1.5 ml reaction tubes

Centrifuge 5810R (Eppendorf, UK)

Used to centrifuge FACS tubes, 15 ml and 50 ml centrifuge tubes

Centrifuge Sorvall® Legend T (Kendro Laboratory Products, Germany)

Used to centrifuge PCR and microarray plates

Freezing container, Nalgene® Mr Frosty (Nalgene, Cat # 5100-001, Thermo Scientific, USA)

Dynatech MR7000 microplate reader (Dynatech Technologies, Billingham, UK)

Magnetic stirrer (Cat # PS6001, Electrothermal Engineering, UK)

BD FACS Aria I flow cytometer (BD Biosciences, Ca, USA)

### 2.1.2 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, San Diego, California, USA). [www.graphpad.com](http://www.graphpad.com)

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Microplate Manager® Software version 6.1 (Bio-Rad Laboratories, Inc.USA)

BD FACSDiva™ software v6.0 (BD Biosciences, Ca, USA)

### 2.1.3 Reagents and general consumables

Product code and manufacturer	
<b>General consumables</b>	
48 well culture plate	Cat # 677102, Greiner Bio-one, Germany
24 well culture plate	P/N 3524, Corning Inc, USA
50 ml Polypropylene centrifuge tubes	P/N 430290, Corning Inc, USA
15 ml Polypropylene centrifuge tubes	P/N 430791, Corning Inc, USA
50 mls Polystyrene multipurpose containers	Cat # 219175, Greiner Bio-One, UK
Polypropylene reaction tubes (0.5 ml, 1.5 ml)	various
MagNA Lyser tubes (and caps)	Cat # CP5518 (Cat # CP5525B), Alpha laboratories, UK
cryogenic vials (nalgene®) from Aug 2009 – Dec 2011	Cat # 5000-0020, Nalge Nunc Int. Corp. USA
cryogenic vials (2 mls) Dec 2011 onwards	P/N 430488, Corning Inc, USA
RadialJaw™ 2.4 mm biopsy forceps	Cat # 13951621, Boston Scientific, Ma, USA
cell strainer (100 µm)	Cat # 352360, BD Biosciences, UK
Cellstar® tissue culture flasks, 25 cm <sup>2</sup>	Cat # 690160, Greiner Bio-one, Germany
Cellstar® tissue culture flasks, 75 cm <sup>2</sup>	Cat # 658170, Greiner Bio-one, Germany
Stir bar	
<b>Media</b>	
DMEM AQmedia™ 4500mg/l glucose L-alanyl-glutamine NaHCO <sub>3</sub>	P/N D0819, Sigma®, UK
HBSS w/o Ca or Mg	Cat # BE10-543F, Lonza, UK
RPMI 1640 AQmedia™ L-alanyl-glutamine NaHCO <sub>3</sub>	P/N R2405, Sigma®, UK
RPMI 1640 L-alanyl-glutamine NaHCO <sub>3</sub>	P/N R8758, Sigma®, UK
DEPC treated water	Cat # PIM9906, Lot # 1009021, Invitrogen, Ca, USA
Bovine Foetal Calf Serum (FBS)	Cat # 10500, Gibco®, UK
PBS w/o Ca or Mg	Cat #. BE17-516F, Lonza
PBS (x10) w/o Ca or Mg	Cat # 70011-036, Gibco®, UK
DMEM 4500mg/l glucose pyridoxine NaHCO <sub>3</sub>	P/N D5671, Sigma®, UK
DPBS	Cat # 14190240, Gibco®, UK
<b>Cell Culture</b>	
Gentamycin (Cidomycin®) 40 mg/ml	Sanofi Aventis
Penicillin / Streptomycin solution, 5000u / 5 mg strep per ml	Cat # DE17-60BE, Lot # OMB206, Lonza
Amphotericin B (Fungizone™) 50 mg / 50,000 u	E.R. Squibb and sons Ltd, UK
Phytohaemagglutinin (PHA)	P/N 19017, Sigma-Aldrich®
<b>RT-qPCR (TaqMan™)</b>	
Taqman® MiRNA Reverse transcription kit	P/N 4366596, Applied Biosystems™, USA

	Product code and manufacturer
100 mM dNTPs (with dTTP)	Lot # 1003037, P/N 4367381, Applied Biosystems™, USA
MultiScribe™ Reverse Transcriptase, 50 U/μL	Lot # 1011098, P/N 4308228, Applied Biosystems™, USA
10x Reverse Transcription Buffer	Lot # 1004081, P/N 4319981, Applied Biosystems™, USA
RNase Inhibitor, 20 U/μL	Lot # P02429, P/N 4308224, Applied Biosystems™, USA
TaqMan® Universal Master Mix, AmpErase® UNG, (x2) contains; AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference, and optimized buffer	P/N 4304437, Applied Biosystems™, USA
Random hexomers	Lot # N12470, P/N 58002113-a , Applied Biosystems™, USA
Hsa-miR-155	P/N 4427975, assay id 002623, Applied Biosystems™, USA
RNU44	P/N 4427975, assay id 001094, Applied Biosystems™, USA
has-miR-194	P/N 4427975, assay id 000493, Applied Biosystems™, USA
hsa-miR-31	P/N 4427975, assay id 002279, Applied Biosystems™, USA
hsa-miR-223	P/N 4427975, assay id 002295, Applied Biosystems™, USA
hsa-miR-146b-5p	P/N 4427975, assay id 001097, Applied Biosystems™, USA
hsa-miR-200b	P/N 4427975, assay id 002251, Applied Biosystems™, USA
hsa-miR-375	P/N 4427975, assay id 000564, Applied Biosystems™, USA
hsa-miR-422a	P/N 4427975, assay id 002297, Applied Biosystems™, USA
MicroAmp™ Optical 384 well reaction plate	P/N 4309849, Applied Biosystems™, USA
ABI PRISM® Optical Adhesive Covers	P/N 4311971, Applied Biosystems™, USA
TSLP	Cat # 4331182, P/N Hs00263639_m1, Applied Biosystems™, USA
GAPDH	P/N 4352934, Applied Biosystems™, USA
β-Actin	P/N 4352667, Applied Biosystems™, USA
<b>RT-qPCR (SYBR®Green)</b>	
RT nanoscript kit	Cat # RT nanoscript, Primer Design Ltd, UK
Oligo d(T) primer Random nonamer primer	
qScript enzyme	
qScript 10X reaction buffer	
RNase/DNase free water	
dNTP mix (10mM)	
DTT (100mM)	
Power SYBR® Green PCR master mix contains; DMSO, Glycerin, Tris-HCl, Sodium Azide	Lot # 1108294, P/N 4367659, Applied Biosystems™, USA
<b>miRNA array</b>	
TaqMan® Universal Master Mix, No AmpErase® UNG, (x2)	P/N 4324018, Applied Biosystems™, USA
Megaplex™ RT Primers, Human pool A v2.1	P/N 4399966, Applied Biosystems™, USA
TaqMan® Array Human MiRNA A Cards v2.0 (4 pack)	P/N 4398977, Applied Biosystems™, USA
TaqMan® Array Human MiRNA A Cards v2.0 (4 pack)	P/N 4398965, Applied Biosystems™, USA
<b>RNA extraction (TRIzol®)</b>	
TRIzol® Reagent solution	Cat # 15596018, Invitrogen, Ca, USA
Ceramic beads (1.4mm)	QBZ-58A , Quackenbush company
Glycogen, stock concentration 20 mg/ml,	P/N 10901393001, Roche
QIAzol® lysis reagent	Cat # 79306, QIAGEN sciences, USA
Chloroform	Cat # C2432, Sigma®, UK
isopropanol	Cat # 19516, Sigma®, UK
<b>RNA extraction (RNeasy)</b>	
RNeasy plus mini kit	Cat # 74134, QIAGEN sciences

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Product code and manufacturer	
<b>Mouse experiments</b>	
TNBS (1M)	Cat # 92823, Sigma-Aldrich®, UK
Oxazolone	Cat # 862207, Sigma-Aldrich®, UK
DSS salt (36–50 x10 <sup>4</sup> Daltons)	Cat # 0216011080, MP Biomedicals
Hypnorm	Vm21757/4000, VetaPharma Ltd
Heparin (5000 iu/ml)	P/N 45774, PC pharmaceuticals
Sno202	Cat # 4427975, assay id 001232, Applied Biosystems™, USA
mus-miR-155	Cat # 4427975, assay id 002571, Applied Biosystems™, USA
mus-miR-125	Cat # 4427975, assay id 002508, Applied Biosystems™, USA
<b>Lymphocyte isolation</b>	
EDTA	Cat # E0511, Melford, UK
Percoll®	P1644, Sigma®, UK
Ficoll-Paque	Cat # 17-1440-03, GE Healthcare
Trypan blue	T8154, Sigma®,UK
Collagenase 2	Cat # C6885, Sigma-Aldrich®, UK
Collagenase 4 (280u/mg)	P/N 41C12537, Worthington, UK
Lithium Heparin BD Vacutainer®	Ref 367377, LH PST2, 8 mls
DMSO	Cat # D8418, Batch # 056K01731, Sigma-Aldrich®, UK
Trypsin	Cat # BE02-007E , Lonza
<b>Transfection</b>	
INTERFERin™ siRNA transfection reagent	Cat # 409-10, Lot # 05INF2309F , Polyplus transfection
Anti-miR™ miRNA inhibitor Negative Control #1	Cat # 17010, Ambion®
has-miR-31 Anti-miR™ miRNA inhibitor (5 nM)	Cat # AM17000, ID # AM11465, Ambion®
<b>ELISA</b>	
Human TSLP Immunoassay kit	Cat # DTSLP0, R&D Systems Inc, USA
TSLP Microplate	P/N 893574
TSLP Conjugate	P/N 893575
TSLP Standard	P/N 893576
Diluent RD1X	P/N 895121
Calibrator Diluent RD6-10	P/N 895468
Wash Buffer Concentrate	P/N 895003
Colour Reagent A	P/N 895000
Colour Reagent B	P/N 895001
Stop Solution	P/N 895032
Plate Covers	
<b>FACS (surface antibody staining)</b>	
FACS tubes. 5 ml polystyrene round bottom tube	ref 352054, BD Falcon™
PE conjugated anti-human CD3	Cat # 21270034, Immunotools
Pacific blue conjugated anti-human CD4 Clone OKT4	Cat # 11-0048-73, eBioscience
Allophycocyanin conjugated Anti-human CD3, Clone UCHT1	Cat # 17-0038-71, eBioscience
PE conjugated anti-human CD25 Clone BC96	Cat # 12-0259-42, eBioscience
PE conjugated anti human CD3	Cat # 21270034, Immunotools
<b>FACS (FoxP3, intracellular staining)</b>	
FITC conjugated FoxP3, clone 236A/E7	Cat # 11-4777-73, eBioscience
IC Fixation Buffer	Cat # 00-8222-49, eBioscience
Permeabilization Buffer (10X)	Cat # 00-8333-56, eBioscience

## 2.1.4 Media receipts

Unless indicated otherwise the following media receipts are used.

## 1 mM EDTA

Additives	strength	volume	Final concentration
Ca <sup>2+</sup> and Mg <sup>2+</sup> free HBSS		485 mls	
Penicillin / Streptomycin	5000 u/ml, 5 mg/ml	10 ml	1000 u/ml, 100 µg/ml
Gentamycin	40 mg/ml	1.25 ml	100 µg/ml
Amphotericin	250 µg/ml	2 ml	1.0 µg/ml
EDTA	0.5 mM, pH 7.4	1 ml	1 mM

## HBSS

Additives	strength	volume	Final concentration
Ca <sup>2+</sup> and Mg <sup>2+</sup> free HBSS		485 mls	
Penicillin / Streptomycin	5000 u/ml, 5 mg/ml	10 ml	1000 u/ml, 100 µg/ml
Gentamycin	40 mg/ml	1.25 ml	100 µg/ml
Amphotericin	250 µg/ml	2 ml	1.0 µg/ml

## RPMI

Additives	strength	volume	Final concentration
RPMI 1640		435 mls	
L-glutamine			
Sodium bicarbonate			
Penicillin / Streptomycin	5000 u/ml, 5 mg/ml	10 ml	100 u/ml, 100 µg/ml
Gentamycin	40 mg/ml	1.25 ml	100 µg/ml
Amphotericin	250 µg/ml	2 ml	1.0 µg/ml
FBS		50 mls	10%

## RPMI 1640 AQmedia™

Additives	strength	volume	Final concentration
RPMI 1640 AQmedia™		440 mls	
L-alanyl-glutamine			
Sodium bicarbonate			
Penicillin / Streptomycin	5000 u / 5mg per ml	10 ml	100 u/ml, 100 µg/ml
FBS		50 mls	10%

## DMEM AQmedia™

Additives	strength	volume	Final concentration
DMEM AQmedia™		440 mls	
4500mg/l glucose			
L-alanyl-glutamine			
Sodium bicarbonate			
Penicillin / Streptomycin	5000 u / 5 mg per ml	10 ml	100 u/ml, 100 µg/ml
FBS		50 mls	10%

## Percoll gradient

Percoll gradient	volume	Ingredients
100%	25 mls	22.5 mls Percoll + 2.05 mls PBS (x10) +0.45 mls CMF-HBSS
60%	10 mls	6 mls 100% Percoll + 4 mls CMF- HBSS
40%	10 mls	4 mls 100% Percoll + 6 mls CMF-HBSS

## 2.2 Samples

### 2.2.1 Patient selection

Patients were recruited from the endoscopy department of the University Hospital Southampton (UHS), UK<sup>1</sup>. Peripheral blood and intestinal biopsies were obtained from consenting patients aged 18 years or older who had an established diagnosis of either UC or Crohn's using standard criteria (Mowat et al., 2011).

Patients were excluded if they had comorbidity requiring the use of immunosuppressive or immune altering medication, excluding inhaled therapy for asthma. Patients with PSC or other undiagnosed liver abnormalities were excluded.

No surgical samples were used as patients undergoing resection represent a distinct phenotype, which are likely to have immunological differences from those patients undergoing endoscopy.

Control samples were obtained from healthy individuals with a macroscopically and histologically normal intestine. Control subjects were attending endoscopy for investigation of intestinal bleeding, abdominal pain, polyp surveillance or elective surveillance following cancer resection.

### 2.2.2 Clinical scoring

Prior to endoscopy peripheral blood was taken for; Full Blood Count which includes the Haemoglobin (Hb) and haematocrit (Hct), urea, creatinine (Cr) and serum electrolytes, liver function, which locally includes serum albumin, aspartate transaminase, total protein, alkaline phosphatase and bilirubin, C reactive protein (CRP) and erythrocyte sedimentation rate (ESR). Biochemical and haematology tests were performed by the pathology laboratory at UHS, and results processed and recorded in the patient notes for use in clinical management. Clinical parameters including; pulse and temperature were recorded as part of routine nursing care.

The clinical severity of UC was scored using the Truelove and Witts criteria (Table 2, page 7) and the Mayo score (Table 3, page 8). The clinical severity of Crohn's

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<sup>1</sup> UHS is a 800 bed tertiary referral inner city university hospital with a gastroenterology service catering for approximately 600,000 population, with an estimated 3400 patients with IBD. SGH performed 2991 colonoscopies between 1<sup>st</sup> January and 31<sup>st</sup> December 2011 inclusive.

was scored using the CDAI (Table 7, page 12) and Harvey Bradshaw Index (Table 8, page 13). Scores were calculated by the author using Microsoft Office Excel 2007, Microsoft Corporation, USA.

### 2.2.3 Endoscopic scoring

The absence of macroscopic inflammation, (endoscopic healing) is rapidly becoming the desired outcome for both clinical trials and clinical treatment. The degree of endoscopic activity either at diagnosis or following treatment is associated with disease prognosis, treatment failure and need for surgery. However many of the scoring systems are complex, impractical, not widely used and because of adaption and/or methodology difficult to compare across studies, see the SES-CD, Table 9, page 14.

The definition of normal and inflamed mucosa differs between studies making comparison difficult. This thesis uses the following definitions: Active inflammation is defined as macroscopic inflammation at the site of biopsy. Inactive UC or inactive Crohn's is defined as an established diagnosis of UC or Crohn's in the region of the intestine from which the biopsy is taken but there is no macroscopic or microscopic (see section 2.2.4) evidence of inflammation at the site of biopsy, or anywhere within the colon, at the time of biopsy. Unaffected mucosa is defined as a biopsy taken from an area of normal mucosa at a time when there is actively inflamed mucosa elsewhere within the intestine. The reason for making these distinctions is that IBD is a systemic disease with a systemic cytokine response, so even if the mucosa is macroscopically normal at the site of biopsy it is being influenced by a milieu of systemic inflammatory cytokines, so cannot be considered to be in a non-inflammatory environment.

Endoscopic appearance and activity was scored by myself using the Baron score (Baron et al., 1964) for both UC and Crohn's. For Crohn's the absence or presence of ulceration at the site of biopsy was also noted.

### 2.2.4 Microscopic scoring

Histological analysis was performed in a separate paired mucosal biopsy sample sent to the histology department at UHS. The histology samples were scored using

## **Chapter 2. Materials and Methods**

**Geboes score 0 - 5 (Geboes et al., 2000). A Geboes score of 0 was deemed to be normal or without features of current inflammatory activity.**

### **2.2.5 Sample collection**

**Biopsies were taken using 2.4 mm bite biopsy forceps (RadialJaw™4 biopsy forceps, Boston Scientific, USA). Biopsies were either placed in cryogenic vials and immediately frozen in liquid nitrogen prior to storage at -80°C, or placed in processing media as described for individual experiments.**

## 2.3 Lymphocyte extraction

Together lymphocytes of the villous epithelium and lamina propria are called mucosal lymphocytes. Within each biopsy there will be two distinct lymphocyte subsets, the intraepithelial lymphocyte and the lamina propria lymphocyte. In health CD8<sup>+</sup> T cells predominate in the epithelial layer and CD4<sup>+</sup> in the lamina propria (Selby et al., 1981), in IBD the total number of T cells increases but the ratio of CD4<sup>+</sup> : CD8<sup>+</sup> remains the same (Selby et al., 1984).

Inflammatory bowel disease is a condition of the mucosa, so for the purposes of this thesis lymphocytes of the epithelium and lamina propria were extracted from biopsies and combined.

### 2.3.1 Extraction of mucosal lymphocytes from biopsies

Firstly, the following media is prepared; 1mM EDTA, HBSS, RPMI and Percoll® gradients as outlined in section 2.1.4.

Between 6 and 8 biopsies are immediately placed into a multipurpose container with 20 mls of 1 mM EDTA and gently stirred at 37°C for 25 minutes. Taking care not to decant visible tissue, the media is decanted into a 50 ml centrifuge tube and diluted 1:1 with HBSS and kept on ice whilst another 20 mls of 1 mM EDTA is added to the biopsies and stirred again at 37°C for 25 minutes. This process is repeated 3 times in total. The decanted media (x3) are centrifuged at 1500 rpm at 18°C for 10 minutes. The supernatant is discarded and the pellets are re-suspended in 10 mls of HBSS, passed through a cell strainer, and pooled together. The solution is made up to 40 mls with HBSS and placed on ice. To the remaining biopsy tissue add 20 mls RPMI, supplemented with 10% FBS and 1 mg/ml collagenase type 4, stir at 37°C. After 30 minutes add 20 mls of HBSS and pass the solution through a cell strainer. The resulting solution, along with the solutions previously pooled together, are centrifuged at 1500 rpm at 18°C for 10 minutes. Supernatants are discarded, and the 2 separate pellets are combined and dissolved in 4 mls 40% Percoll.

A Percoll gradient of 3 discontinuous layers consisting of 100%, 60% and 40% Percoll respectively is created by careful layering 2 mls of each concentration in 2

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separate 15 ml centrifuge tubes, Figure 9. The 40% Percoll contains the mucosal lymphocytes. The Percoll gradients are centrifuged at 1500 rpm at 18°C for 25 minutes, to achieve isopycnic banding of cells between the layers of Percoll. Collect and combine the lymphocyte layers and mix with 10 mls HBSS. Centrifuge at 1500 rpm at 4°C for 10 minutes. Discard the supernatant and add 10 mls of HBSS. Centrifuge at 1500 rpm at 4°C for a further 10 minutes. After discarding the supernatant re-suspend the pellet in 1 ml of RPMI + 10% FBS.

Lymphocytes can be counted (section 2.3.3, p73), frozen for storage (section 2.3.4, p73) or used directly.

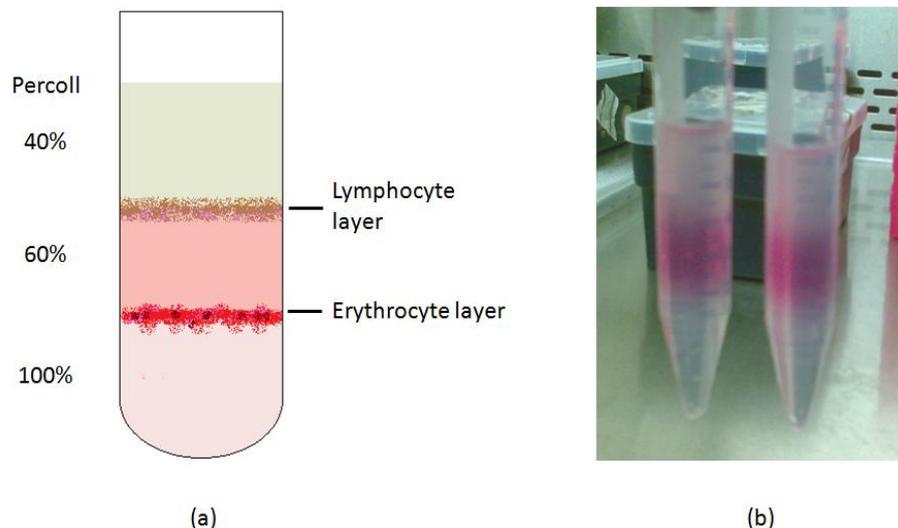


Figure 9. Percoll® gradient for lymphocyte extraction from biopsies. (a) A schematic showing the isopycnic layering of cells after centrifuging the Percoll® gradient, (b) the Percoll® gradient before centrifuging, the 40% Percoll® (top layer) is cloudy due to the suspended lymphocytes, debris and other cells.

### 2.3.2 The extraction of PBMCs from whole blood

Blood is taken directly into a 8 ml Lithium Heparin vacutainer to prevent thrombin formation. When ready, dilute blood in an equal volume of PBS at room temperature. Place 15 mls Ficoll-Paque in a 50 ml centrifuge tube and carefully layer 15 mls of the blood / PBS mixture onto the top. Centrifuge at 2000 rpm at 18°C for 20 minutes. Remove the resulting serum layer and transfer the white

cloudy PBMC layer into a sterile 15 ml centrifuge tube. Add twice the volume ( $\approx 10$  mls) of PBS and centrifuge at 1500 rpm at 18°C for 15 minutes. Discard the supernatant. Add a further 10 mls PBS and centrifuge at 1500 rpm at 18°C for 15 minutes. Discard the supernatant, and re-suspended the pellet in RPMI + 10% FBS, aim for a cell concentration of  $0.5 - 1.0 \times 10^6$  cells per ml.

PBMCs can be counted (section 2.3.3, p73), frozen for storage (section 2.3.4, p73) or used directly.

In health PBMCs consist of approximately 75% T cells, 10% Monocytes, 10-15 % B cells, and about 1-2 % Dendritic cells (Autissier et al., 2010). The proportion of cell subset is donor and disease specific.

### 2.3.3 Cell count and viability analysis

Combine 50  $\mu$ l of trypan blue suspension (0.4%) with 50  $\mu$ l of cell solution. Live cells are impermeable to Trypan blue and thus can be differentiated from dead cells. Cells in the resulting solution can be counted using a haemocytometer. The mean of two separate counts are used.

Cell viability is calculated using the following equation;

$$\frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100 = \text{viability (\%)}$$

### 2.3.4 Cell and Lymphocyte storage

Firstly prepare the freezing media; Combine 9.5 mls FBS with 0.5 mls DMSO at room temperature in a 15 ml centrifuge tube.

After counting with the haemocytometer, centrifuge the cell solutions at 1500 rpm at 18°C for 5 minutes to form a pellet. The pellet is re-suspended in freezing media at a concentration of  $5.0 - 10.0 \times 10^6$  cells per ml. After allowing 15 minutes for the media to enter the cells, place cell solutions in cooled cryogenic vials, and then into a freezing container containing 100% isopropyl alcohol. Place the freezing container into a -80°C freezer. The freezing container ensures that the

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cells are cooled slowly at  $\leq 1^{\circ}\text{C}$  per minute to minimise cell damage. On the following day, transfer cryogenic vials to liquid nitrogen for permanent storage.

## 2.4 RNA extraction

Total RNA was extracted from both cell solutions and whole biopsies using either TRIzol® reagent or the RNeasy™ plus mini kit. These are examples of a liquid-liquid and column (silica) based extraction techniques respectively. Both methods use guanidine isothiocyanate (GITC) to lyse cells and denature proteins, RNases and DNases.

The TRIzol® protocol relies on the phase separation between nucleic acids which are dissolved in the upper aqueous phase, and proteins (dissolved in phenol) and lipids (dissolved in chloroform) which are dissolved in the organic phase. Under acidic conditions DNA is dissolved in the interphase or lower phase. RNA is then precipitated out of the aqueous phase using isopropanol.

The RNeasy™ protocol relies on the properties of RNA binding to silica at differing salt and buffer conditions. Although more expensive an advantage of a column based techniques is the absence of chloroform and phenol which can be potentially hazardous and unpleasant.

### 2.4.1 RNA extraction using TRIzol®

Whole biopsies require to be homogenised initially. First place whole biopsies straight from frozen into a MagNA Lyser tube containing 1 ml TRIzol® reagent and 1 g ceramic beads. Homogenize twice using MagNA Lyser at 6300 rpm for 40 seconds, cool on ice between steps. Place the resulting homogenate into 1.5 ml reaction tubes.

Cell solutions are first pelleted by centrifuging at 1500 rpm at 18°C for 5 minutes. Decant the supernatant and add the pellet to 1 ml TRIzol® in a 1.5 ml reaction tube. Stand at room temperature for 30 minutes.

To each TRIzol® / sample mixture add 0.2 mls chloroform (≈20% by volume) and vortex for 15 seconds. Leave to settle. Centrifuge at 13,200 rpm at 4°C for 40 minutes. Draw off the clear aqueous phase and place in 1.5 ml reaction tubes. Add 1 ml isopropanol to each sample and vortex for 10 seconds. Add 5 µl of glycogen to each sample and vortex again for 15 seconds. Leave at room temperature for 10

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minutes. Store the samples at -80°C overnight. The following day, thaw samples on ice and centrifuge at 13,200 rpm and 4°C for 30 minutes. Remove the isopropanol leaving the glycogen pellet to which add 900 µl of ice cold 75% alcohol. Leave for 10 minutes. Centrifuge again at 13,200 rpm and 4°C for 30 minutes. Remove the alcohol and allow the residual liquid to evaporate, take care not to over dry the pellet. Add 50 µl of RNA free water to create the stock RNA solution.

Store the stock RNA at -80°C until required.

### 2.4.2 RNA extraction using RNeasy™

The total RNA from the murine models of colitis was extracted using an RNeasy™ Plus mini kit, according to manufacturer's instructions.

In a MagNA Lyser tube containing 1 g ceramic beads add 20 mg tissue and 600 µl RLT Buffer plus (which contains GITC and 6 µl β-mercaptoethanol). Homogenise the mixture using a MagNA Lyser at 6400 rpm for 40 seconds. Cool on ice and repeat previous step. Place the homogenate into 1.5 ml reaction tubes and centrifuge at 13,200 rpm and 18°C for 3 minutes. Apply the supernatant onto the gDNA Eliminator spin column and centrifuge at 10,000 rpm and 18°C for 30 seconds. Add an equal volume of 70% ethanol and apply 500 µl of the resulting mixture on the RNeasy spin column. The RNA is absorbed to the silica gel membrane of the spin column by centrifuging at 10,000 rpm and 18°C for 15 seconds. Repeat the step x3 and discard the flow through. Apply wash buffers to remove contaminants; to do this first apply 700 µl of Buffer RWI and centrifuge at 10,000 rpm and 18°C for 20 seconds followed by two applications of the Buffer RPE and the same process. The RNA is then eluted into a new 1.5 ml reaction tube by adding 30 µl nuclease free water, waiting for 2 minutes then centrifuging at 10,000 rpm and 18°C for 1 minute.

The stock RNA is stored at -80°C until required.

### 2.4.3 Quantification of RNA

A 2 µl aliquot of each RNA sample was analysed using a ND-1000 NanoDrop® Spectrophotometer using manufacturer's instructions. The concentration of RNA

was calculated by analysing the absorbance of UV light at a wavelength of 260nm. Each reading was performed in duplicate. If duplicates differed by >10% the RNA sample was mixed again with a pipette and RNA concentrations re-calculated. The 260nm / 280nm absorbance ratio was used to assess purity and samples used only if ratio >1.8.

## 2.5 Reverse Transcription - quantitative PCR

### 2.5.1 Introduction

Reverse Transcription – quantitative PCR (RT - qPCR) is a highly sensitive method of quantifying gene expression by measuring the expression of mRNA in a sample. It is a 2 step process involving first reverse transcription (RT) of RNA into complementary DNA (cDNA) and then amplification and quantification of cDNA (qPCR). The process is done in real time (real time – qPCR) which allows quantification of the cDNA and therefore quantification of the original RNA.

#### 2.5.1.1 Reverse transcription

Reverse transcription is a process whereby mRNA is reverse transcribed into cDNA by reverse transcriptase. The poly (A) tail at the 3' end of the mRNA attracts the RT primer which can either be oligo dTs, random primers or gene specific primers each of which have their own characteristics. The RT primer provides the 3'-OH group which is necessary for initiation of cDNA synthesis. Triggered by the 3'OH group, reverse transcriptase uses the mRNA as a template and by adding individual nucleotides at the 3' end builds a single cDNA strand, Figure 10. At this stage there is a mRNA:cDNA hybrid, and because RNA contains Uracil in place of Thymine, the original mRNA strand must be replaced to create the desired double stranded cDNA (ds cDNA). The mRNA strand is digested using the enzyme RNase, followed by DNA Polymerase which builds the other strand of the ds cDNA using the remaining mRNA pieces on the mRNA:cDNA hybrid as the template. The result of reverse transcription is ds cDNA.

The general principles of reverse transcription need special consideration under certain situations. MiRNAs are too short, approx. 17-24nt, for standard quantitative PCR which requires a transcript (cDNA) length of more than twice the forward and reverse primers, >40nt. miRNAs also do not contain a polyp (A) tail like mRNA. The specially designed miRNA stem loop primers overcome these two deficiencies by binding to mature miRNA in place of the Poly (A) tail and adding length during the RT reaction. Extra length and a higher melting temperature are also provided by a “hooked” tail on the forward primer during the qPCR reaction. During the reverse

transcription reaction, the RT primer / RNA duplex results in >100 times improved efficiency compared with traditional linear RT primers, and greater discrimination between similar miRNAs and precursor miRNAs through base stacking and spatial constraints (Chen et al., 2005). The Looped RT primer is disrupted by the reverse primer during qPCR.

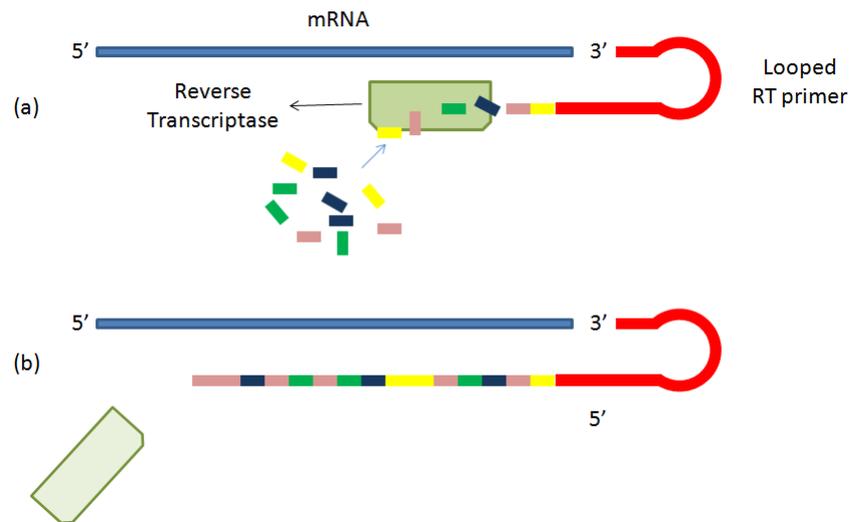


Figure 10. Reverse Transcription (RT), the initial step in which a mRNA/DNA hybrid is created, adapted from TaqMan™ microRNA assays: Protocols (2006).

#### 2.5.1.2 Real Time – qPCR

Real Time – qPCR is a process which identifies, amplifies and quantifies DNA targets by utilising a thermostable DNA polymerase to increase cDNA to detectable levels.

Repeatedly raising and then lowering the temperature of the reaction breaks the double helix of DNA which allows single stranded primers to anneal to the single strands of DNA. As the solution cools DNA Polymerase synthesises new DNA strands (Polymerization) by adding nucleotides commencing from the primer. Each time the process is repeated the number of target DNA sequences doubles so that after 40 cycles there are over 1 billion copies of the original sequence.

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DNA quantification relies on the activation and measurement of a fluorescent product. There are 2 ways in which this is achieved; probe based (e.g. TaqMan™) or by intercalator / dsDNA binding (e.g. SYBR Green®, Lux® primers and hybridisation probes).

TaqMan™ real time qPCR requires a pair of specific forward and reverse primers and a specific MGB (minor groove binder) probe<sup>1</sup>. The probe consists of a fluorophore reporter (6-carboxyfluorescein, FAM) which is covalently attached to the 5' end of an oligonucleotide probe, a non-fluorescent quencher (NFQ) and a minor groove binder (MGB) attached to the 3' end of the probe. The probe is designed to anneal to the DNA within a region between the forward and reverse primers and whilst the whole probe is intact and the NFQ is in close proximity to the reporter, fluorescence is suppressed by Förster-type energy transfer.

During polymerization the Taq polymerase moves from the 5' end towards the 3' end building the nascent strand by adding nucleotides and extending the primer. On reaching the probe the exonuclease activity of the Taq polymerase hydrolyzes the phosphodiester bonds releasing the probe from the DNA template and breaking the probe into pieces releasing the reporter, Figure 11.

Free from the suppression of the NFQ, fluorescence emitted by the reporter can be detected and quantified by the PCR cyclor.

The Intercalator based method utilizes the light absorbance changes and characteristics of a non-specific DNA binding dye, SYBR Green® as it binds non-specifically to dsDNA. The main advantage of SYBR® Green is the non-specific RT reaction which means the cDNA can be used for multiple gene targets, another advantage is the cheaper cost. However, it is less accurate than probe based real time PCR which emits fluorescence only after hybridization of the probe with its specific target DNA compared SYBR® Green which can report non-specific products such as primer dimers.

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<sup>1</sup> TaqMan™ MGB probes take their name from the thermostable Taq Polymerase and videogame PacMan which was popular at the time that this technique was developed and which shares a common action of slicing up the enemy (hydrolyzing the MGB probe in this case).

In this thesis I have used both probe based RT-qPCR (TaqMan™) and an intercalator (SYBR Green®) methods. The protocols for miRNA (TaqMan™) and mRNA (TaqMan™ and SYBR Green®) differed and are therefore considered separately.

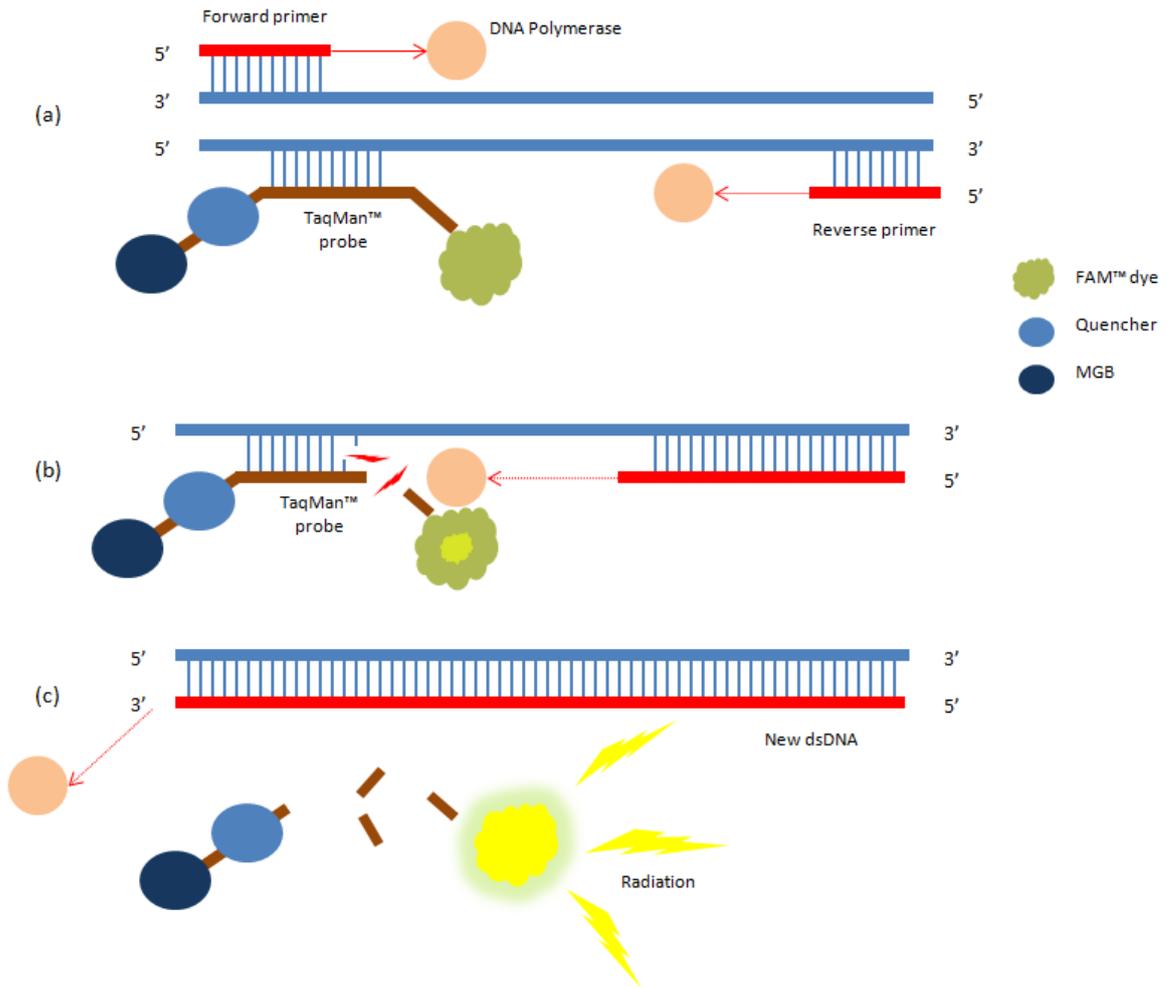


Figure 11. Quantifying dsDNA using TaqMan™ MGB probes. (a) The TaqMan™ probe anneals to the RNA between the forward and reverse primers. (b) On reaching the probe the Taq polymerase hydrolyses the phosphodiester bonds releasing the probe and reporter. (c) Free from the NFQ, the reporter emits fluorescence.

### 2.5.1.3 Gene quantification

PCR reactions for both miRNAs and mRNAs were ran on the 7900HT Fast Real-Time PCR System (Applied Biosystems™, USA) and analysed using the ABI PRISM® 7900HT Sequence detection system (SDS) v2.3 software (Applied Biosystems™,

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USA, copyright 2005). The threshold was calculated automatically for all samples but checked manually to ensure the threshold had been set at an appropriate level within the exponential phase of the PCR. The cycle number at which the fluorescence crossed the threshold value is called the Ct value. Samples were analysed in triplicate where possible. Calculations were only performed for those samples with 2 or more reliable Ct values. Ct values were ignored if; the amplification plot was erroneous or the Ct value for duplicate samples was >1 cycle difference.

Gene expression was calculated using the comparative Ct ( $2^{-\Delta\Delta Ct}$ ) method (Schmittgen and Livak, 2008), Figure 12. The comparative method calculates the relative difference (fold difference) in gene expression between the sample and a control.

The first step in the calculation is to normalise the Ct value for the gene of interest (GOI) within each sample against a constitutively expressed endogenous control (the house keeper gene),  $\Delta Ct$ . This is done to correct for differences in the amount of cDNA introduced at the start of the reaction for each sample. The house keeper genes used were RNU44, MammU6,  $\beta$ -ACT, sno202 or GAPDH. The second step is to calculate the  $\Delta\Delta Ct$ , which is the difference in the  $\Delta Ct$  between each sample and the average  $\Delta Ct$  for all controls. The  $\Delta\Delta Ct$  is incorporated into the final calculation,  $2^{-\Delta\Delta Ct}$ , which results in the relative fold difference, or relative expression in the GOI between the sample and a control sample.

$$\begin{aligned} \text{Fold difference} &= 2^{-\Delta\Delta Ct} \\ \Delta Ct_{\text{sample}} - \Delta Ct_{\text{control}} &= \Delta\Delta Ct \\ Ct_{\text{gene of interest (sample)}} - Ct_{\text{house keeper gene (sample)}} &= \Delta Ct_{\text{sample}} \\ Ct_{\text{gene of interest (control)}} - Ct_{\text{house keeper gene (control)}} &= \Delta Ct_{\text{control}} \end{aligned}$$

Figure 12. Derivation of the  $2^{-\Delta\Delta Ct}$  calculation

## 2.5.2 Probe based miRNA RT-qPCR (TaqMan™)

TaqMan™ miRNA reverse transcription reaction and RT-qPCR are adaptations of the manufacturers protocol (Applied Biosystems, 2006).

### 2.5.2.1 Reverse Transcription protocol

Firstly prepare stock RNA and master mix. Thaw stock RNA and dilute 1  $\mu$ l with an appropriate volume of ice cold RNA free water to achieve a concentration of 10 ng/ $\mu$ l. To prepare the master mix combine on ice the ingredients outlined in Table 16. All ingredients were purchased from Applied Biosystems™ and volume multiplied in each experiment by the number of samples studied.

Master mix	No. Samples x ( $\mu$ l)	Applied Biosystems™ Part numbers (unless otherwise stated)
100mM dNTPs (with dTTP)*	0.075	4367381
MultiScribe™ Reverse Transcriptase, 50 U/ $\mu$ L*	0.500	4308228
10x Reverse Transcription Buffer*	0.750	4319981
RNase Inhibitor, 20 U/ $\mu$ L*	0.094	4308224
RNase free water	4.081	Lot # 1009021, Invitrogen
GOI RT primer	1.500	See Table 17
House keeper gene RT primer	1.500	See Table 17

Table 16. Master Mix for miRNA Reverse Transcription (\* individual components of the Taqman® MiRNA Reverse transcription kit part # 4366596 Applied Biosystems™, USA). GOI = gene of interest.

After thoroughly mixing the master mix with a pipette, combine 7  $\mu$ l of master mix with 0.5  $\mu$ l (5 ng) diluted RNA in a 0.5 ml reaction tube. There will be a reaction tube for each sample and one for a negative control (0.5  $\mu$ l H<sub>2</sub>O) multiplied by the number of primers being investigated. The Reverse Transcription reaction is ran on a Tetrad™ 2 thermal cycler with the following thermal conditions; 30 minutes at 16°C, 30 minutes at 42°C then 5 minutes at 85°C.

The primer assays used for experiments are shown in Table 17.

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House keeper gene	
RNU44 (human)	part # 4427975, assay id 001094
Sno202 (mouse)	Cat # 4427975, assay id 001232
Gene of interest (GOI)	
Hsa-miR-155	part # 4427975, assay id 002623
has-miR-194	part # 4427975, assay id 000493
hsa-miR-31	part # 4427975, assay id 002279
hsa-miR-223	part # 4427975, assay id 002295
hsa-miR-146b-5p	part # 4427975, assay id 001097
hsa-miR-200b	part # 4427975, assay id 002251
hsa-miR-375	part # 4427975, assay id 000564
hsa-miR-422a	part # 4427975, assay id 002297
mus-miR-155	Cat # 4427975, assay id 002571
mus-miR-125	Cat # 4427975, assay id 002508

Table 17. TaqMan™ MiRNA assays. Each assay includes one solution used in the RT reaction containing a specific miRNA RT primer, and one solution used in the real time PCR reaction which contains a miRNA specific forward primer, the same miRNA specific reverse primer and a miRNA specific TaqMan MGB probe. All probes purchased from Applied Biosystems™, USA.

### 2.5.2.2 Real time - qPCR

Firstly prepare master mixes. A master mix is created for each GOI and housekeeper gene with the ingredients in Table 18.

	Product info.	( $\mu$ l)
TaqMan® Universal PCR Master Mix	Applied Biosystems, USA, P/N 4304437	5.0
Nuclease free water		3.1
PCR Primers	Table 17	0.5

Table 18. The recipe for miRNA PCR master mix. A separate master mix is created for each primer. Each qPCR reaction is performed in triplicate for the housekeeper gene and duplicate for the GOI. Volumes of reagents are multiplied thrice (or twice for duplicates) the number of samples to be analysed

Into separate wells of a MicroAmp™ Optical 384 well reaction plate combine 8.6 µl of master mix and 1.4 µl of the corresponding cDNA sample. Each PCR reaction is ran in triplicate for the housekeeper and duplicate for the GOI. Cover the PCR plate with an adhesive cover. Centrifuge at 2000 rpm and 18°C for 5 minutes.

The PCR reaction is ran 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.

The amplification of GOI is normalised to RNU44 for human samples and sno202 for mouse samples and compared with control samples using the ABI PRISM® 7900HT Sequence detection system (SDS) v2.3 software. Relative expressions of miRNAs in samples were analysed by the  $\Delta\Delta C_t$  method.

### 2.5.3 Probe based mRNA RT-qPCR (TaqMan™)

#### 2.5.3.1 Reverse Transcription

Two hundred ng of total RNA is mixed with 1.0 µl Reverse Transcription Buffer (10x), 0.4 µl dNTPs (100 mM, with dTTP), 1.0 µl random hexamers, 0.5 µl MultiScribe™ Reverse Transcriptase (50 U/µL), 0.5 µl RNase Inhibitor (20 U/µL) and made up to 10.0 µl with RNase / DNase free water.

The RT reaction is ran on the Tetrad™ 2 thermal cycler with the following conditions; 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes.

#### 2.5.3.2 Real Time - qPCR

Firstly; dilute the cDNA to a ratio of 1:10 with nuclease free water and create a PCR master mix for each PCR primer. The PCR master mixes contain 2.5 µl TaqMan® Universal PCR Master Mix, 1.0 µl nuclease free water and 0.25 µl of the PCR primer. The primers used for this type of RT-qPCR are shown in Table 19.

Each PCR reaction is performed by adding 3.75 µl of the PCR master mix to individual wells of a MicroAmp™ Optical 384 well reaction plate and adding 1.25 µl of diluted cDNA.

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Each sample is performed in triplicate for the house keeper and duplicate for the GOI. Non template controls are ran replacing the primers with nuclease free water. Cover the reaction plate with an adhesive cover.

Centrifuge the reaction plate at 2000 rpm at 18°C for 5 minutes and run the PCR reaction 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.

The amplification of the GOI is normalised to GAPDH and compared with control samples using the ABI PRISM® 7900HT Sequence detection system (SDS) v2.3 software. Relative expressions of the GOI in samples were analysed by the  $\Delta\Delta C_t$  method.

House keeper gene	
GAPDH	P/N 402869
Gene of interest (GOI)	
TSLP	Cat # 4331182

Table 19. Primers used in TaqMan mRNA RT-qPCR reactions. Purchased from Applied Biosystems unless otherwise stated.

### 2.5.4 Intercalator based mRNA RT-qPCR (SYBR® Green)

#### 2.5.4.1 Reverse Transcription reaction

Reverse transcription is performed in a two-step process using the Precision qScript<sup>1</sup> Reverse transcription kit and following the manufacturers protocols (Primer Design Ltd, 2005).

The annealing step of the RT reaction consists of creating a pair of reaction tubes for each RNA sample containing the following ingredients; 1  $\mu$ l Oligo dT primer, 1  $\mu$ l Random nonamer primer and 1  $\mu$ g of total RNA, each reaction volume is made

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<sup>1</sup> Now known as “nanoscript”

up to 10  $\mu\text{l}$  with nuclease free water. The resulting mixture is placed in the Tetrad™ 2 thermal cycler (Bio-Rad Life Sciences, USA) for 65°C for 5 minutes, to eliminate secondary structure in the RNA that can interfere with cDNA synthesis.

For the extension step of the RT reaction the following ingredients are added to each pair of reaction tubes with (RT+) or without (RT-) the reverse transcriptase enzyme (1.0  $\mu\text{l}$  qScript enzyme); 1.0  $\mu\text{l}$  dNTP mix (10 mM), 2.0  $\mu\text{l}$  DTT (100 mM) and 2.0  $\mu\text{l}$  qScript buffer (10x). The reaction mixtures are made up to 20  $\mu\text{l}$  with nuclease free water.

The annealing step is ran on the Tetrad™ 2 thermal cycler at 25°C for 5 minutes then at 55°C for 20 minutes followed by heat inactivation of the enzyme at 75°C for 15 minutes.

Thus, each RNA sample is either reverse transcribed into a single strand cDNA (RT+) or subjected to the same process but without reverse transcription indicating that any cDNA formed is derived from contaminating DNA (RT-).

### 2.5.4.2 Quantitative PCR amplification

The cDNA is diluted to a concentration of 1:10 with nuclease free water.

For each sample, into separate wells of a MicroAmp™ Optical 384 well reaction plate, 2.5  $\mu\text{l}$  aliquots of the diluted RT+ cDNA (x4 wells) and 2.5  $\mu\text{l}$  aliquots of the diluted RT- cDNA (x2 wells) are combined with 5.0  $\mu\text{l}$  *Power SYBR Green* PCR master mix and 2.0  $\mu\text{l}$  of nuclease free water. Into two of the RT+ cDNA reactions 0.5  $\mu\text{l}$  of a solution containing the GOI sense and antisense oligonucleotide primers (Table 20) are added, and into the other 4 wells, 2 containing RT+ cDNA and 2 containing RT- cDNA, 0.5  $\mu\text{l}$  of  $\beta$ -actin sense and antisense oligonucleotide primers are added (Table 20). The total reaction volume is 10.0  $\mu\text{l}$ .

The reaction is therefore performed in duplicate with  $\beta$ -actin RT+ as a control, and  $\beta$ -actin RT- as a no RT control.

Primers were purchased by PrimerDesign Ltd and reconstituted with nuclease free water to a stock concentration of 4.5  $\mu\text{M}$ , resulting in a working concentration of

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300 nM. However, due to the formation of primer-dimers the stock primers are diluted further as indicated in Table 20, and explain in paragraph 2.5.4.3.2.

The real-time PCR is ran on the 7900HT Fast Real-Time PCR System (Applied Biosystems™, USA) using the following thermal cycling conditions; stage 1, 50°C for 2 minutes followed by 95°C for 10 minutes. Stage 2, 95°C for 15 seconds followed by 60°C for 1 minute, stage 2 is repeated for 40 cycles.

The amplification of the GOI is normalised to  $\beta$ -actin and the relative expression of the GOI compared with control samples using the  $\Delta\Delta C_t$  method.

GENE		Optimised primer dilution ratio	Product length	T <sub>m</sub>	GC%	Sequence
$\beta$ ACT (house keeper)		1:1	Supplied by PrimerDesign Ltd			
IL-4	Amplicon		111	76.7		
	Sense	1:4		57.0	55.0	AACGAGGTCACAGGAGAAGG
Antisense				57.0	55.0	CACCTTGAAGCCCTACAGA
IL-5	Amplicon		128	74.5		
	Sense	1:1		56.9	57.9	ATGGACGCAGGAGGATCAC
Antisense				56.7	41.7	TTGAAGTTAGATAGGAGCAGGAAG
IL-10	Amplicon		100	72.0		
	Sense	1:1		57.1	41.7	CATACTGCTAACCGACTCCTTAAT
Antisense				57.1	52.4	GGGCATCACTTCTACCAGTA
FOXP3	Amplicon		127	79.1		
	Sense	1:6		58.5	52.4	AGGACAGACCACACTTACTGC
Antisense				58.7	63.2	CCAGTGGCAGCAGAAGGTG
TNF- $\alpha$	Amplicon		94	73.7		
	Sense	1:4		57.2	50.0	GCCTCCCTCTCATCAGTTCTAT
Antisense				56.8	52.6	TTTGCTACGACGTGGGCTA
INF- $\gamma$	Amplicon		128	74.5		
	Sense	1:4		58.0	40.0	TGATTACTACCTTCTTCAGCAACAG
Antisense				57.5	63.2	CTGGTGGACCACTCGGATG
TGFB1	Amplicon		113	92.6		
	Sense	1:1		55.8	50.5	TGGACACACAGTACAGCAAG
Antisense				55.5	55.0	GTAGTAGACGATGGGCAGTG

Table 20. Primers used in SYBR® Green RT-qPCR reactions. All primers are mus primers purchased from PrimerDesign Ltd.

2.5.4.3 Trouble shooting Intercalator based mRNA RT-qPCR (SYBR® Green)

2.5.4.3.1 Contaminating DNA

Because SYBR® Green is a non specific DNA binding dye, contamination of the PCR reaction by DNA will lead to an amplification product in RT- reaction, Figure 13. The assumption is that the contaminating DNA is genomic DNA (gDNA) as this is often impossible to completely eliminate from preparations. To determine if the PCR amplification due to the gDNA is sufficiently negligible compared with the cDNA template to include in the analysis, the Ct values of the RT+ and RT- reactions were compared in all experiments using SYBR® Green. Only reactions in which the Ct for the control RT- was >10 cycles greater than the Ct for the control RT+, are analysed.

Assuming 100% efficiency where 1 Ct equals a 2 fold difference, a RT- control sample with a Ct value 10x greater than a RT+ control sample will contain ( $2^{\Delta Ct}$ ) 1024 fold less of the target sequence, indicating that approximately 0.1% of the amplification of the target gene overall is due to contamination. In the example depicted in Figure 13, the amplification plots for the house keeper  $\beta$ -Actin are shown. The contamination of the sample is 0.000002%.

*Calculations*

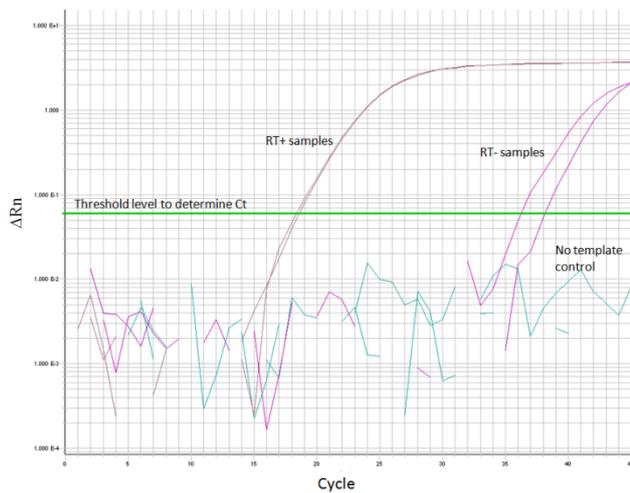
RT+ = 18.25

RT- = 37.20

$\Delta Ct = 18.95$

Fold difference =  $2^{\Delta Ct} = 2^{18.95}$

Fold difference =  $5.1 \times 10^5$



(a)

(b)

Figure 13. Trouble shooting contaminating cDNA in RT-qPCR (a) Fold difference between Ct values (b) Contaminating DNA.  $\beta$ -Actin expression in murine colonic mucosa. Ct value for the RT+ is 18.25, and for the RT- reaction 37.20.

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In no experiments was the contamination of gDNA > 0.001%, and therefore it was unnecessary to consider extracting and purifying RNA using an alternative method, or was it thought necessary to add a DNase treatment step into the protocols.

### 2.5.4.3.2 Optimising primer concentration

DNA binding dyes used during PCR lack specificity because they bind indiscriminately to the dsDNA products formed during a PCR reaction which then adds to the overall fluorescent signal. Artefacts commonly include primer-dimers which are the result of hybridization between primer pairs, and less commonly spurious amplification products. Melting curve analysis uses the melting characteristics of DNA to identify unwanted PCR products.

All primers for SYBR® Green PCR experiments are purchased from Eurogentec at a concentration of 250 nM. By performing SYBR® Green real time qPCR with a variety of primer dilutions, optimal dilutions are identified in which amplification of the desired products occurs but primer dimers did not occur Figure 14. This was carried out for all primers, Table 20 p88.

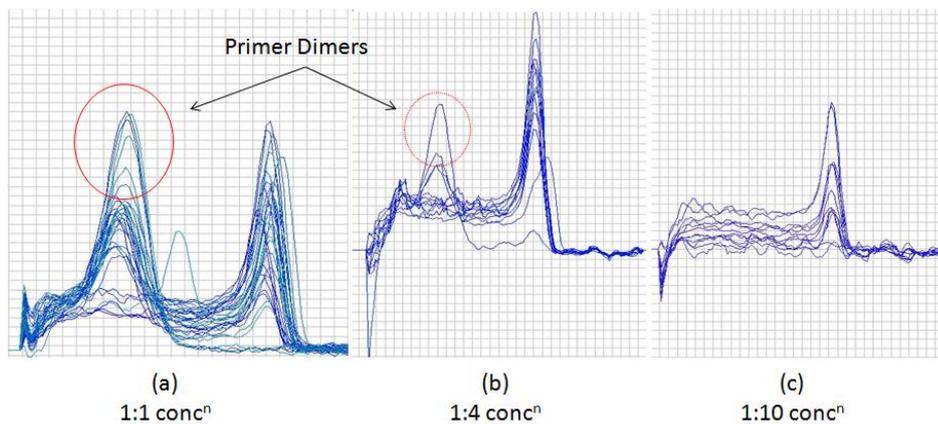


Figure 14. Primer Dimers. The melting curve of 3 experiments (a-c) in which the concentration of TGF- $\beta$  primers is 250 nM (a), 62.5 nM (b) and 25 nM.

## 2.6 TaqMan® miRNA Array

### 2.6.1 Introduction

The TaqMan® Human MiRNA array A card is a microfluidic 384 well plate containing dried TaqMan primers and probes capable of measuring 377 of the most commonly expressed human miRNAs, along with 4 control genes and a negative control.

The reverse transcription reaction and TaqMan® Low Density Array are performed following the manufacturers protocols; Megaplex™ Pools Protocol, and Applied Biosystems TaqMan® Low Density Arrays on 7900HT Real-Time PCR Systems.

For Practical reasons only Human Pool A was analysed, and the following adaptations to the manufacturers protocol were made; adaption 1. For each array card 250 ng of total RNA was taken from 2 samples and combined. Combining RNA from separate samples serves two functions; the first reduces the inter-sample variation in miRNA levels, the second reduces the cost of the reaction. Adaption 2. Array cards were ran in pairs, a disease and control card. The reason for this is that array results were thought to be more reliable when comparisons were made between array cards that were ran in close proximity. Adaption 3. Analysis was made using ranking rather than relative fold change, see section 2.6.4.

### 2.6.2 Megaplex™ reverse transcription reaction

The Megaplex™ RT reaction includes 500 ng total RNA mixed with the components of the TaqMan® MiRNA Reverse Transcription Kit and Megaplex™ RT Primers, Human pool A, v2.1 to the ratios shown in Table 21. The total volume of the reaction is 7.5 µl made up with nuclease free water.

The resulting mixture was placed in the Tetrad™ 2 thermal cycler under the following thermal cycling conditions; stage 1, 16°C for 2 minutes followed by 42°C for 1 minute followed by 50°C for 1 second. Stage 1 was repeated 40 times. Stage 2, 85°C for 5 minutes.

## Chapter 2. Materials and Methods

RT reaction mix components		( $\mu$ l)
TaqMan® MiRNA Reverse Transcription Kit	(P/N 4366596)	
• dNTPs with dTTP (100 mM)		0.2
• MultiScribe™ Reverse Transcriptase (50 U/ $\mu$ L)		1.5
• 10 $\times$ RT Buffer		0.8
• RNase Inhibitor (20 U/ $\mu$ L)		0.1
• MgCl <sub>2</sub> (25 mM)		0.9
Megaplex™ RT Primers, Human pool A, v2.1	(P/N 4399822)	0.8
Nuclease-free water		0.2
Total		4.5

Table 21. Megaplex™ RT reaction mix. Components purchased from Applied Biosystems unless otherwise stated.

### 2.6.3 TaqMan™ low density MiRNA array

For the TaqMan MiRNA Array, combine 6.0  $\mu$ l of cDNA with 450.0  $\mu$ l TaqMan® Universal Master Mix, No AmpErase® UNG, (x2). Make up to 900.0  $\mu$ l with nuclease free water. Dispense 100  $\mu$ l into each port of the TaqMan® Human MiRNA array A card, as shown in Figure 15.

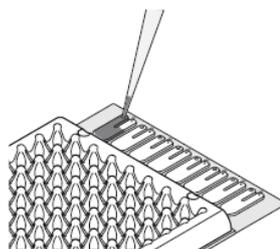


Figure 15. Array card, inserting the master mix. Picture taken from manufacturer's instructions (Applied Biosystems, 2006).

Seal the card and centrifuge twice at 1200 rpm for 1 minute. The reaction is run using the 7900HT Fast Real-Time PCR System using the 384 well TaqMan Low Density Array default thermal-cycling conditions.

### 2.6.4 Analysing Arrays

The array cards were analysed using RQ manager version 1.2 and the manufacturer's protocol, Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantification Using Comparative CT Getting Started Guide.

Threshold values for each miRNA were set manually within the exponential slope of the amplification plot. Ct  $\leq 35$  were considered significant. Raw Ct values were exported to Microsoft Excel 2010 and relative expression ( $2^{-\Delta\Delta Ct}$ ) calculated. The expression of miRNAs are normalised to the mean MammU6 for each microarray card and compared with healthy control. The standard equations outlined in Figure 12 are used.

There is an option of 3 constitutively expressed endogenous controls for the TaqMan™ low density miRNA array cards, RNU44, RNU48 and MammU6. Mammalian U6 amplifies in 4 wells, retrospective analysis of preliminary experiments shows that MammU6 has a more consistent Ct value within and across array cards (3 cards; active sigmoid UC, active sigmoid Crohn's and healthy controls shown in Figure 16.) compared with RNU44 and RNU48. The difference between the lowest and highest Ct value for MammU6, RNU44 and RNU48 is 0.5034, 0.7143 and 0.6576 respectively.

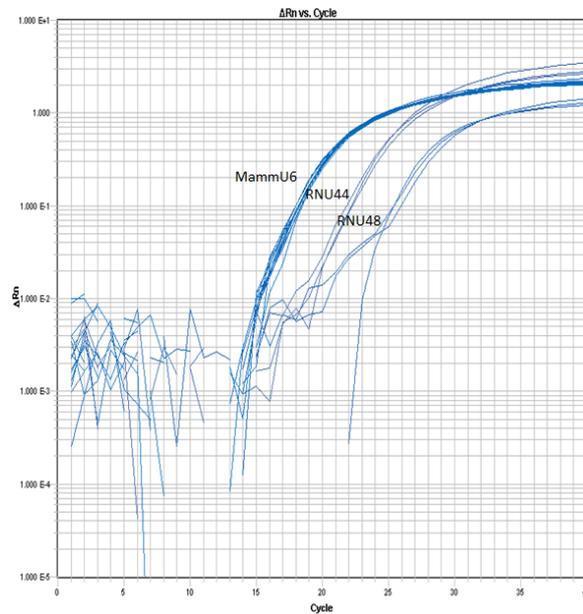


Figure 16. TaqMan low density miRNA array card endogenous controls.

## 2.7 Bioinformatics: The search for potential mRNA / miRNA interactions

### 2.7.1 Introduction

The application of computer science in biology, “Bioinformatics”, has become an invaluable tool for miRNA gene detection and miRNA target prediction.

The first miRNA was reported in 1993 (Lee et al., 1993, Wightman et al., 1993) and the first human miRNA 7 years later (Pasquinelli et al., 2000). Based mainly on evolutionary conservation early predictions suggested there would be approximately 200 human miRNAs (Lim et al., 2003) but within a few years evolutionary non-conservation was included and the predicted number of miRNAs increased to approximately 800 (Bentwich et al., 2005). By the middle of the decade computational software helped increase the number of predicted miRNA to over 1000 (Berezikov et al., 2005).

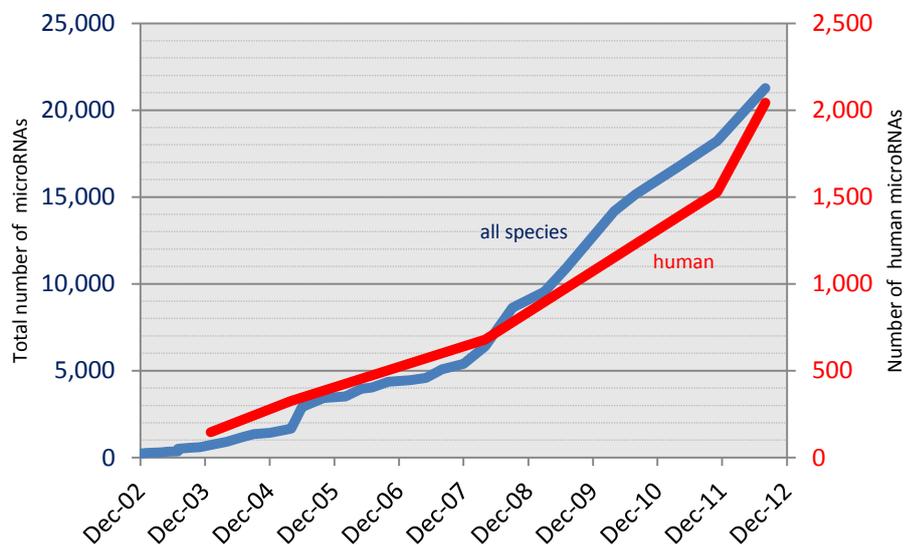


Figure 17. The number of known miRNAs. Adapted from the miRBase Sequences database, release 19, August 2012. (red = human, blue = all).

It is clear that the predicted number and identification of miRNAs is determined by the methodology used and attempts to predict the number of miRNA is very quickly surpassed by the number of miRNA that are identified, currently there are 2042 miRNAs listed on the miRBase Sequences database, release 19 (Griffiths-Jones et al., 2008), a number which is doubling every few years, Figure 17. There is no current consensus at what value this will peak.

The complete miRBase sequences database now on release 19 (August 2012) and available at [www.mirbase.org](http://www.mirbase.org)<sup>1</sup>, currently contains 21,264 hairpin precursor miRNA entries, expressing 25,141 mature miRNAs, in 193 species, and is considered the primary repository of miRNA sequences.

### 2.7.2 MiRNA target prediction

MiRNA target predictions show that each miRNA is expected to have approximately 200 gene targets (Lewis et al., 2003) although this is largely dependent on the computational program which is used. Mir-31 for example has 678 conserved targets and another 265 poorly conserved targets using TargetScan version 6.0 (Lewis et al., 2003, Garcia et al., 2011), 279 targets using MirTarget2 (Wang and El Naqa, 2008, Wang, 2008) but only 159 targets that are conserved in vertebrates with PicTar (Grun et al., 2005), databases accessed 10<sup>th</sup> January 2012.

Taking into account the overlap between miRNA and their predicted targets it is thought that about 1/3 of human protein coding genes, currently between 25,000 and 30,000, fall under the influence of one or more miRNAs, although to date the number of proven associations is measured in the 100s.

One of the weaknesses of bioinformatics target prediction is the poor overlap between computational programs because of the different features that each algorithm incorporates to calculate potential binding (Min and Yoon, 2010), Table 22. The features used for the different algorithms fall into a number of categories; Base pairing, thermodynamic stability, comparative sequence analysis and presence of multiple target sites.

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<sup>1</sup> Previously the Sanger database (<http://miRNA.sanger.ac.uk>)

## Chapter 2. Materials and Methods

Name of program	Name of algorithm	features	reference
TargetScan release 6.0 (www.targetscan.org)	TargetScan	An algorithm which calculates a "context+" score. See 2.7.3.1. Mainly seed region pairing and complementary	(Lewis et al., 2003)
PicTar (www.pictar.mdc-berlin.de)	PicTar	Thermodynamic stability	(Grun et al., 2005)
miRDB (www.mirdb.org)	MirTarget2	Support vector machine (SVM)	(Wang, 2008, Wang and El Naqa, 2008)
Microcosm Targets version 5 (www.ebi.ac.uk) (accessed 27 <sup>th</sup> May 2011)	miRanda	Formerly miRBase Targets, it is a web resource developed by the Enright Lab at the EMBL-EBI. Main focus is complementary	
MiRNA.org (www.miRNA.org)	miRanda	August 2010 release. Complementary.	(Enright et al., 2003)
RNAhybrid (www.bibiserv.techfak.uni-bielefeld.de/rnahybrid)	RNAhybrid	Thermodynamic stability and statistical method including minimum free energy hybridisation	
DIANA microT (www.diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi)	DIANA microT	Thermodynamic stability	
	MiRanda	Uses a number of steps; the first is the miRanda algorithm to identify complimentary sites which are scored between 0 and 100, where 0 represents no complementarity and 100 represents complete complementarity. The scoring is weighted in favour of complementarity at the 5' end of the miRNA. Complete complementarity is required in the seed region (bases 2-7 of the miRNA 5'). The next step is to assess secondary structure and thermodynamic stability using Vienna RNA folding routines, followed by a final step to ensure target site conservation across at least 2 species.	

Table 22. Computational methods for miRNA target prediction

### 2.7.2.1 Base pairing

The importance of base pairing originated from the initial observation that the target site of the lin-14 UTR is complementary to the 5' region of the lin-4 miRNA (Lee et al., 1993). Target site prediction programs now categorise, or score, base pairing depending on the pattern of predicted binding of which there are a

number; 1. 5' end dominant canonical, i.e. perfect base pairing at the 5' end of the miRNA and extensive base pairing through to the 3' end, 2. Seed only, i.e. base pair matching at the 5' end of the miRNA but not elsewhere and 3. Compensatory, i.e. where bases are not perfectly matched in the seed region but are extensively matched through to the 3' end.

### 2.7.2.2 Thermodynamic properties

The thermodynamic properties of the miRNA:mRNA hybrid is calculated using a number of RNA folding programs such as the Vienna, RNAfold or Mfold programs which calculate the Gibbs free energy ( $\Delta G$ ) of the hybrid binding. Despite this approach being included in a number of algorithms, notably the miRanda algorithm, it appears to be insensitive and non-specific because of the low free energy involved with miRNA:mRNA hybridization (Watanabe et al., 2007).

### 2.7.2.3 Comparative sequence analysis

This is the comparison between orthologous 3' UTR sequences in humans with other species, on the basis that miRNA and their targets are highly conserved. Comparative sequence analysis reduces the false positive results, but unfortunately also increases false negatives.

### 2.7.2.4 Presence of multiple target sites

Approximately 40% of miRNA genes are within the introns or exons of protein coding genes and are therefore co-expressed and suspected to influence the expression of those genes. Multiple miRNA target sites in the 3' UTR of the mRNA may influence target repression, and is therefore taken into account in some computational programs.

## 2.7.3 Available methods

There are now several dozen miRNA target prediction programs most of which are freely available online. Descriptions are given in Table 22.

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### 2.7.3.1 Target scan (release 6.0)

In the initial release of TargetScan it was required to have complete base pairing in the seed region of the miRNA, this was followed by a complex calculation including thermodynamic properties of the mRNA:miRNA hybrid and finally conservation of the miRNA target across species (Lewis et al., 2003). There have been many step wise adaptations between the first version of this program and the latest version released in November 2011 (version 6.0), the most prominent being the change in the accepted base pairing patterns. The initial version required perfect complimentary base pairing in the seed region, position 2-8 of the 5' end miRNA (denoted as 7mer), from version 4.0 onwards slightly different seed region base pairing patterns denoted 8mer, 7mer-m8 and 7mer-1A were included, Table 23. The most recent adaptations include seed pairing stability and target site abundance factors which also effect the robustness of miRNA targeting and reduce the false negative rate (Garcia et al., 2011).

8mer	An exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'
7mer-m8	An exact match to positions 2-8 of the mature miRNA (the seed + position 8)
7mer-1A	An exact match to positions 2-7 of the mature miRNA (the seed) followed by an 'A'

Table 23. TargetScan base pairing patterns.

TargetScan now ranks predicted targets using the “context+” score, which is the sum of the following features; site-type contribution, 3' pairing contribution, local AU contribution, position contribution, target site abundance contribution and seed-pairing stability contribution. The calculation is complex but explained by Garcia et al. (Garcia et al., 2011). The relevance of the stepwise changes to TargetScan algorithm is that it is difficult to compare scores, or target gene ranking from one version to the next, and with the addition of new steps to the algorithm, the number of predicted target genes is expanding.

TargetScan (v6.0) analyses 18,393 genes for potential targeting.

### 2.7.3.2 miRDB (version 4.0)

miRDB is a freely available online resource for miRNA target prediction and annotation, it tests the miRNAs listed in the miRBase sequences database release 18 against the genes listed in the PANTHER (Protein ANalysis THrough Evolutionary Relationships) database, available at <http://www.pantherdb.org>.

MiRNA target predictions are calculated using MirTarget2 algorithm. MirTarget2 is based on support vector machines (SVMs) which incorporate novel features that are important to target down-regulation. Support vector machines are a statistical method in which the algorithm is able to categorise complex patterns into separate groups by generalizing the information of known examples and creating hypothetical differences between them, then placing the new pattern, or miRNA sequence, into one of these groups. Support vector machines are a form of artificial intelligence, in which the computer algorithm learns to process new data, without the need for new programming.

The MirTarget2 algorithm gives a score between 1 and 100, but states that a score >80 is likely to be true and supplementary evidence is needed for those targets with scores <60.

### 2.7.3.3 Method used

Bioinformatic searches were performed using a combination of TargetScan version 5.2 (TargetScan algorithm) and miRDB version 4.0 (MirTarget2 algorithm). There is no universally accepted ideal algorithm, and there are no head to head trials. These 2 separate bioinformatics tools search for targets in 2 distinctly different ways, by using a combination of two separate methods it is envisaged that the target gene search will be a more sensitive method for identifying potential targets.

## 2.8 Experimental models of colitis

### 2.8.1 Introduction

All animals are maintained in accordance with local and national regulations. Methods adhered to previously published protocols (Wirtz et al., 2007).

The method of sedation involved firmly holding the mouse by the scruff of the neck between the index finger and thumb and inverting in the palm of the hand. Performed correctly the mouse is still, calm and held firm. The mouse is sedated with 125 µl intraperitoneal (i.p.) hypnorm, 25 µg fentanyl and 1.25 mg fluanisone (Vm21757/4000, VetaPharma, UK). The mouse is then returned to the cage and monitored. Depending on absorption, after a short time the mouse is still, does not move away when touched and ready to be used.

	Strain	Age	Sex		Model
AM18	C57 BL/6	8 weeks	F	Oral water	Day 0-6, water Day 7, harvest
AM29	C57 BL/6	8 weeks	F	2.0% TNBS	Day 0, sensitisation Day 4, 2% TNBS Day 8, harvest
AM32	C57 BL/6	8 weeks	F	2.5% TNBS	Day 0, sensitisation Day 4, 2.5% TNBS Day 8, harvest
AM35	C57 BL/6	6.5 weeks	F	3.0% TNBS	Day 0, sensitisation Day 4, 3% TNBS Day 8, harvest
AM24	C57 BL/6	8 weeks	F	2% Oxazolone	Day 0, 2% Oxazolone Day 3, harvest Day 5, harvest
AM22	C57 BL/6	8 weeks	F	2% DSS	Day 0-6, 2% DSS Day 7, harvest

Table 24. Chemical induced animal models of colitis.

Insertion of chemicals and / or the carrier ethanol is achieved by inserting a 3.5F flexible plastic catheter to 4 cm proximal to the rectum whilst the mouse is sedated. This is generally easily achieved although care is taken not to injure the mouse colon. After infusion the mouse is laid down in a head down position to

prevent chemicals from leaking from the rectum. Care is taken to avoid leakage as oral ingestion of chemical may lead to tolerance.

Mice are closely monitored until fully recovered from the sedation. The mice are then weighed daily. Animal models are denoted “AM”, the timelines for each of the models are listed in Table 24.

### 2.8.2 TNBS

Trinitrobenzene Sulfonic acid (TNBS) is mixed with 50% ethanol. A variety of TNBS concentrations are used to induce colitis and compared against 50% ethanol which is used as the control. Calculations for creating the TNBS concentration for insertion are shown in Table 25.

TNBS %	TNBS ( $\mu$ l) (1 M stock solution)	100% ethanol ( $\mu$ l)	Water ( $\mu$ l)	Total volume ( $\mu$ l)
control	0.0	37.5	37.5	75
0.5	1.7	37.5	35.8	75
1.0	3.4	37.5	31.6	75
2.0	6.8	37.5	30.7	75
2.5	8.5	37.5	29.0	75
3.0	10.2	37.5	27.3	75

Table 25. TNBS solution calculations.

On day one, each mouse is sedated then sensitised with a 75  $\mu$ l dose of 0.5% TNBS per rectum, after recovery from the sedation they continue to be housed under the standard conditions.

On day five, each mouse is sedated with 125  $\mu$ l hypnorm and a 3.5F catheter inserted 4 cm proximal to the rectum. Either 75  $\mu$ l of TNBS in 50% ethanol or 75  $\mu$ l of 50% ethanol as control is inserted carefully and the mouse placed head down.

Samples are harvested 4 days later.

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### 2.8.3 Oxazolone

On day one, each mouse is sedated with 125 µl hypnorm. A 3.5 F catheter is inserted 4 cm proximal to the rectum. Mice are either treated with 100 µl of 2% Oxazolone (Cat No. 862207, Sigma-Aldrich®, UK) or 100 µl 50% ethanol for the control. Mice are placed head down and monitored until fully recovered.

Samples are harvested 3 and 5 days later.

### 2.8.4 DSS

Two percent Dextran Sulphate Sodium (2% DSS, Cat. No. 0216011080, MP Biomedicals, UK) supplemented drinking water is created by mixing 40 g of DSS salt in 500 mls drinking water. On day one, normal drinking water is substituted for 2% DSS. The 2% DSS drinking water is renewed on Day 3 and 5. On day 7 the 2% DSS is changed for normal drinking water and on day 8 samples are harvested. Control mice continue to be supplied with normal drinking water.

### 2.8.5 Termination and sample harvesting

The mice are terminated with cervical dislocation and placed immediately supine. Using sterile forceps and scissors, the axillary artery is immediately dissected and blood collected into 1 ml syringes containing 500 units of heparin. (P/N 45774, PC pharmaceuticals, UK).

Immediately following blood collection the superficial layers of the abdomen are dissected revealing the peritoneum. With a 26 G needle 5 mls of Dulbecco's Phosphate Buffered Saline (DPBS, cat No. 14190240, Gibco®) is injected into the peritoneal space and massaged. After 45 seconds the DPBS is aspirated taking care to avoid puncturing the bowel.

Using sterile forceps and scissors a ventral midline incision is made. The colon is exposed and dissected as close to the anus as possible. The colon is measured and characteristics such as ischemia and inflammation noted. The distal 4 cm is removed and divided so that the central 8mm is placed into neutral buffered 10% formalin and stored at 4°C. The proximal 1.6 cm is snap frozen in liquid nitrogen

for protein and PCR analysis and the distal 1.6 mm snap frozen for storage and Western Blot.

### 2.8.6 Determining the degree of inflammatory activity

The degree of inflammatory activity in all models of experimental colitis was measured in 3 ways; the first is by determining the weight change of each mouse daily throughout each experiment. The second is by measuring the weight of the distal 4 cm of resected colon and the third is by visual analysis of the resected colon.

Measuring daily weights serves two purposes; the first is to measure wasting as a pseudo-marker of inflammatory activity and thus use the measured weight to predict when the inflammation is maximal and the best time to harvest tissue (Wirtz and Neurath, 2007). The second purpose is to monitor the welfare of the mice. Although weight loss alone is a poor predictor of wellbeing (Ray et al., 2010) it is still accepted that weight loss > 20% is a criteria for euthanasia and an indication that the experimental design may be too aggressive.

Additional indicators of the degree of colonic inflammation is conducted by a visual inspection for thickening, strictures, dilations and quality of the stool followed by measurement of the weight of the distal 4 cm of colon after harvesting and removal of stool. Together the three measures of inflammatory activity ensured that the experiments were conducted at the optimal time following chemical induction.

## 2.9 Tissue culture and Phytohaemagglutinin stimulation

### 2.9.1 Jurkat cell culture

Jurkat cells are an immortalised line of T Lymphocytes that are commonly used to study T cell signalling. CD4<sup>+</sup> JM22 Jurkat cells were donated by Dr S. Mansour (University of Southampton, UK).

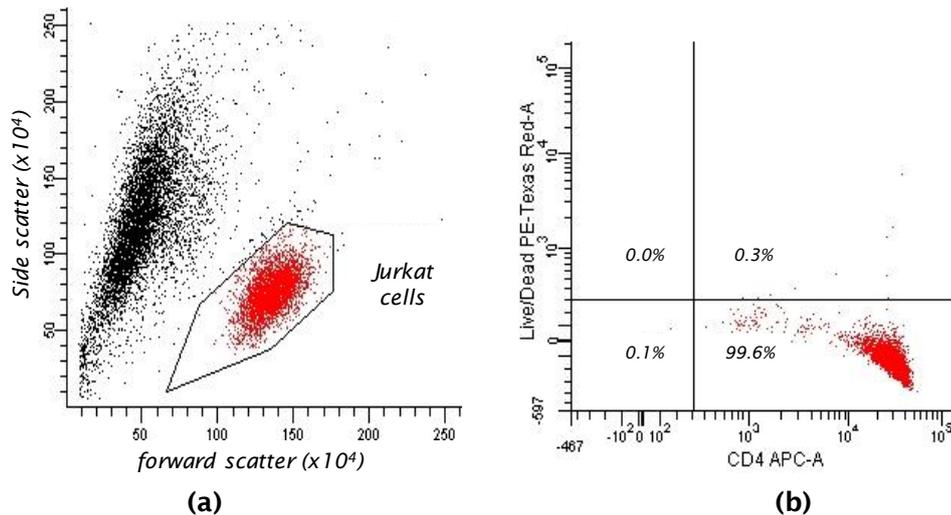


Figure 18. CD4 surface staining of Jurkat cells. (a) Representative sample of cells in Jurkat cell culture, 33.3% of total count are Jurkat cells, shown in red. (b) Representative dot blot of CD4<sup>+</sup> and PI<sup>-</sup> Jurkat cells. Dot blot demonstrates 99.6% of cells are live CD4<sup>+</sup>.

Jurkat cells are maintained in suspension in a 75 cm<sup>2</sup> cell culture flasks. The culture media consists of; RPMI 1640 AQmedia™ supplemented with L-alanyl-glutamine, Sodium bicarbonate, 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cultures are maintained at 37°C humidified air with 5% CO<sub>2</sub>. Media is replaced every 2 to 3 days to maintain the cell concentration at approximately 5.0 - 10.0 x 10<sup>5</sup> cells per ml.

### 2.9.2 THP-1 cell culture

A human acute monocytic leukaemia cell line. Physiologically they behave like monocytes, and are therefore used to mimic macrophage response.

Culture conditions are identical to those of Jurkat cells, section 2.9.1.

### 2.9.3 HT29 cell culture

HT29 are an immortalised intestinal epithelial cell line that behaves like intestinal epithelial cells.

HT29 cells are maintained adhered to a 75 cm<sup>2</sup> cell culture flasks. The culture media consists of DMEM AQmedia™ supplemented with 4500 mg/L glucose, L-alanyl-glutamine, Sodium bicarbonate, 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cultures are maintained at 37°C humidified air with 5% CO<sub>2</sub>. Media is replaced when the cells become confluent.

### 2.9.4 Phytohaemagglutinin

Phytohaemagglutinin (PHA) is a plant lectin, a protein that is evolutionarily conserved for its action on the animal GI tract. Exposure to PHA causes short term disruption and functional impediment to the GI tract epithelium resulting in nausea which discourages animal consumption, giving lectin containing plants a survival advantage. Interestingly longer term exposure leads to epithelial stimulation, maturation and growth through mechanisms including MAPK activation and c-fos mRNA expression (Linderoth et al., 2006, Otte et al., 2001).

Lectins are proteins that bind to carbohydrate moieties on cell surface glycoproteins, PHA gained its name through the ability to agglutinate and clump red blood cells. It functions on immune cells by clustering surface proteins. PHA thus stimulates T lymphocytes through cross linking the TCR and CD3, resulting in a rapid increase cell signalling and RNA synthesis (Kay, 1968), protein synthesis and mitosis (Otte et al., 2001).

The optimum concentration and culture time for PHA varies between experimental conditions and requires optimisation, although studies vary between 0.5 and 10.0 µg/ml, and 2 hours to 1 month depending on the experimental aims.

### 2.9.5 PHA stimulation of PBMCs

PBMCs from the peripheral blood of 3 healthy individuals are extracted using technique described in section 2.3.2. After counting, the PBMCs are diluted in RPMI

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1640 AQmedia™ supplemented with L-alanyl-glutamine, Sodium bicarbonate, 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin at a concentration of  $0.5 \times 10^6$  PBMCs per ml.

Place  $0.5 \times 10^6$  PBMCs into each well of a 48 well plate. Add 0.0, 2.5, 5.0 and 10.0 µg/ml of PHA to individual wells and culture at 37 °C in humidified air at 5% CO<sub>2</sub>. At 0, 24 and 48 hours, draw off culture solutions and place in a 1.5 ml reaction tube. Centrifuge at 1,500 rpm and 18°C for 15 minutes. Discard the supernatant. Wash the pellet by adding 1 ml room temperature PBS and centrifuging again at 1,500 rpm and 18°C for 15 minutes. Discard the supernatant. Dissolve the pellet in 1 ml TRIzol® and store at -80 °C until used for RNA extraction. Experiments are done in duplicate, with 2 wells for each sample at each concentration and each time period.

### 2.9.6 PHA stimulation of HT29 cells

#### 2.9.6.1 Seeding

HT29 are maintained as adherent cultures. First remove culture media from the flask and wash twice by instilling 10 mls of warm PBS, swilling the flask and removing. To release the cells from the culture flasks add 2 mls Trypsin and incubate at 37°C for 15 minutes. Agitate to release cells from the flask, add 10 mls DMEM (plain). Transfer the cell solution to a 15 ml centrifuge tube and centrifuge at 1500 rpm and 18°C for 5 minutes. Discard the supernatant and re-suspend the pellet in 10 mls DMEM (plain). Centrifuge again at 1500 rpm and 18°C for 5 minutes. The supernatant is discarded and the pellet dissolved in an appropriate volume of DMEM AQmedia™ supplemented with 4500 mg/L glucose, L-alanyl-glutamine, Sodium bicarbonate, 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin to achieve a concentration of  $0.5 \times 10^6$  cells/ml. The resulting HT29 cell solution can be seeded onto culture plates.

#### 2.9.6.2 PHA stimulation

HT29 cells are seeded onto a 24 well plate at a concentration of  $0.5 \times 10^6$  cells per ml. Experiments are conducted in duplicate on post confluent cells after 24-48

hours incubation at 37°C and 5% CO<sub>2</sub>. To half of the wells 2.5 µg/ml PHA is added. The cultures are incubated at 37°C and 5% CO<sub>2</sub>.

After 48 hours the supernatants are discarded and 1 ml TRIzol® added to each well. After 10 minutes the mixture is transferred into 1.5 ml reaction tubes and the RNA extracted using the method outlined in section 2.4.

## 2.9.7 PHA stimulation of THP-1 and Jurkat cells

### 2.9.7.1 Seeding

THP-1 and Jurkat cells are taken from culture flasks and centrifuged at 1500 rpm and 18°C for 5 minutes. The supernatant is discarded and the pellet dissolved in an appropriate volume of RPMI 1640 AQmedia™ supplemented with, L-alanyl-glutamine, Sodium bicarbonate, 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin, to achieve a concentration of  $0.5 \times 10^6$  cells per ml. The resulting THP-1 or Jurkat cell solution can now be seeded in a culture plate.

### 2.9.7.2 PHA stimulation

THP-1 or Jurkat cells are seeded into the wells of a 48 well plate at a concentration of  $0.5 \times 10^6$  cells per ml. Experiments are conducted in duplicate. To half of the wells 2.5 µg/ml PHA, is added, and the other half left alone. The cultures are incubated at 37°C and 5% CO<sub>2</sub>.

After 48 hours the cell solutions are transferred into 1.5 ml reaction tubes and centrifuged at 2000 rpm and 18°C for 5 minutes. The supernatants are discarded and pellets re-suspended in 1 ml TRIzol®. After 10 minutes RNA is extracted using the method outlined in section 2.4.

## 2.10 Antagomir based knockdown of miR-31

### 2.10.1 Introduction

The silencing of miRNAs by synthetically engineered oligonucleotides, “antagomirs”, is an effective method to investigate the loss of function effect of miRNA (Kruzfeldt et al., 2005).

This technique uses the principles of short interfering RNA (siRNA). Antagomirs are siRNA which are perfectly complementary to their target miRNA, in this case miR-31 with addition of extra motifs to prevent degradation and allow delivery into the target cells / organs. Antagomirs bind irreversibly and efficiently to their target thus preventing its function and silencing its effect on downstream factors.

“Off target” effects can limit antagomir specificity through their binding to targets with similar base pair sequences as the intended miRNA, these have been largely negated with newer proprietary transfection reagents, such as TRANSFERin™ which are efficient for target miRNAs at concentrations below that at which specificity is compromised (Caffrey et al., 2011).

### 2.10.2 Method

Firstly Jurkat cells need to be taken from culture and seeded at a concentration of  $2.0 \times 10^5$  cells/ml in a 24 well plate as described in section 2.9.7.1.

Sample #	1					
	2					
	3					
	4					
		-	+	-	+	Anti-miR-31
		-	-	+	+	PHA 2.5 µg/ml

Figure 19. Example of a transfection culture plate. There are 4 samples with a single well for each sample under different conditions.

Prepare two master mixes, one for the mir-31 antagomir and the second for the control antagomir. Each master mix consists of 93.75  $\mu$ l RPMI AQmedia™ (plain), 5.00  $\mu$ l INTERFERin™ and either 1.25  $\mu$ l anti-miR-31 (Anti-miR™) or 1.25  $\mu$ l control antagomir (Anti-miR™ miRNA inhibitor negative control).

Vortex each master mix for 10 seconds and incubate for 10 minutes at room temperature. Add 100  $\mu$ l of the master mix to the appropriate wells. An example of an experiment for 4 samples is shown in Figure 19. Culture the plate at 37°C and 5% CO<sub>2</sub>.

After 24 hours, 2.5  $\mu$ g of PHA (2.5  $\mu$ g/ml) is added to the appropriate wells. Culture at 37°C and 5% CO<sub>2</sub>. After 48 hours the cell solution is transferred to 1.5 ml reaction tubes and centrifuged at 1500 rpm and 18°C for 5 minutes.

The Supernatants are transferred to a separate 1.5 ml reaction tube and stored at -80°C. The pellet is re-suspended in warm PBS and centrifuged again at 1500 rpm and 18°C for 5 minutes. Discard the supernatant. One ml TRIzol™ is added to each reaction tube and the sample either used directly, or stored at -80°C until required.

## 2.11 Enzyme Linked Immunosorbent Assay

### 2.11.1 Introduction

Genes come under the influence of many post-transcriptional factors that alter the efficiency of gene translation into protein. Some factors increase gene translation and protein production such as TLR induced hnRNP U expression which stabilises inflammatory cytokine mRNA and increases TNF- $\alpha$  and IL-1 $\beta$  expression (Zhao et al., 2012), and some factors repress gene translation such as miRNAs. Gene expression may therefore not correlate with protein production.

Two commonly used ways to quantify protein concentration are Western Blot and ELISA. Both use binding antibodies but ELISA has a number of advantages over Western Blot including increased sensitivity and accuracy.

A quantitative sandwich enzyme immunoassay was performed using the manufacturer's protocol (Cat # DTSLP0, © 2010 R&D Systems Inc, USA). Minor variations, outlined below, are made to the TSLP standards to ensure measurement of smaller concentrations of TSLP than would otherwise be possible.

### 2.11.2 Methods

#### 2.11.2.1 Cell culture

Cell culture suspensions are collected into 1.5 ml polypropylene tubes and centrifuged at 1500 rpm for 5 minutes at 18°C. The particulate free supernatant for each sample is removed and placed in a separate 1.5 ml polypropylene tube. These are then stored at -80 °C and thawed immediately prior to the experiment.

#### 2.11.2.2 Biopsy

Colonic biopsies are snap frozen in liquid nitrogen and stored at -80°C. Prior to protein extraction the samples are thawed on ice. The samples are then homogenised by four cycles of freeze thawing with liquid nitrogen in 200  $\mu$ L PBS and 8  $\mu$ L protease inhibitor. The samples are vortexed for 10 seconds between each cycle.

The cell solution is transferred to 1.5 ml polypropylene tubes and centrifuged at 13200 rpm for 10 minutes at 4°C. The palette is discarded and the supernatant aspirated for analysis.

#### 2.11.2.3 ELISA

All reagents are brought to room temperature; the reagents contained within the Human TSLP Immunoassay kit are prepared;

- Wash solution is created by mixing 20 mls of wash buffer concentrate with 480 mls distilled water.
- Standard TSLP concentrations are created, first by reconstituting TSLP standard with 1.0 ml distilled water to create a stock solution of 20,000 pg/ml. Then decreasing concentrations of TSLP are created by diluting appropriate volumes of the TSLP stock solution with the calibrator diluent RD6-10. The following concentrations of TSLP are prepared; 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 pg per ml.

Each well of the microplate is precoated with polyclonal antibody against TSLP. To each well add 100 µl assay diluent RDX1. Followed by 50 µl of either the different concentrations of TSLP standard to create a dilution ladder or cell culture supernatant. The microplate is then sealed with an adhesive cover and incubated at room temperature for 2 hours.

After incubation the solution in each well is removed and the wells are washed x4, filling each well with wash buffer and removing, after the final wash the microplate is inverted and blotted against a paper towel to ensure complete removal of liquid. To each well 200 µl of TSLP conjugate is added, the microplate is then resealed with an adhesive cover and incubated for another 2 hours.

The wash step is then repeated. The colour reagent A and B are mixed together in equal volumes, and 200 µl added to each well. The microplate is sealed and incubated once again for another 30 minutes.

After incubation 50 µl of stop solution is added to each well turning the colour instantly from blue to yellow.

## Chapter 2. Materials and Methods

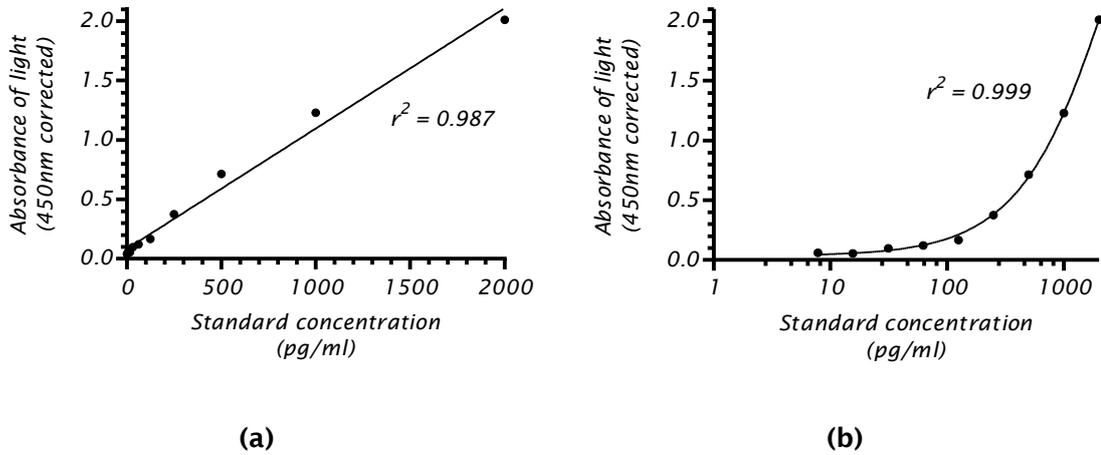


Figure 20. Analysis of the standard curve for ELISA. (a) analysis using linear fit, (b) analysis using 5 parameter logistic regression. Graphs and analysis performed using Microplate Manager® Software.

The optical density of each well is measured at 450 nm with wavelength correction set to 540 nm on the microplate reader. Analysis is performed using Microplate Manager® Software.

It was expected that the concentrations of TSLP in cell supernatants would be low and therefore a five parameter logistic model was used in preference to the linear model to build the standard curve. Its strength is that it more accurately predicts the values at the extreme of the curve, Figure 20b.

## 2.12 Flow cytometry

### 2.12.1 Introduction

Density centrifugation as outlined in section 2.3.1 is reported to yield a cell suspension containing between 80-90% T lymphocytes. This mixed population of lymphocytes and other tissue debris can be further and more accurately quantified and divided using multicolour flow cytometry. Fluorescence activated cell sorting, FACS™, is a method that counts and sorts a heterogeneous mixture of cells into separate populations, it relies on the light scattering characteristics of specific fluorescent labelled antibodies which have been added to and bind to specific antigens expressed by cells within the mixture.

The aim of flow cytometry is to acquire a homogeneous sample of CD3<sup>+</sup>CD4<sup>+</sup> T cells from the lymphocyte mixture extracted from tissue by density centrifugation. These cells are a mixture of both pro-inflammatory and anti-inflammatory T helper cells. The anti-inflammatory subtype is the regulatory T cell which is already known to express a different profile of miRNAs from non T<sub>reg</sub> CD4<sup>+</sup> cells (Rouas et al., 2009). Regulatory T cells can be identified by staining for FoxP3; however this requires cell permeabilization and intracellular staining which prevents analysis of miRNAs. However, it is recognised that the cell surface antigen, CD25, is highly expressed on T<sub>regs</sub>, and that a majority of T<sub>regs</sub> are found in the CD25<sup>high</sup>, or brightly stained, population. The proportion of T<sub>regs</sub> in a population of CD25<sup>low</sup> or CD25<sup>intermediate</sup> cells is generally accepted as low enough to make the assumption that the expression of miRNAs from T<sub>regs</sub> in these populations would not influence the miRNA profile overall, and thus a population of CD4<sup>+</sup>CD25<sup>intermediate</sup> is the activated pro-inflammatory CD4<sup>+</sup> subtype.

For cell surface and intercellular staining a two step process is used which is adapted from the manufacturers FoxP3 staining protocol, “Staining Intracellular Antigens for Flow Cytometry” (eBioscience, 2010). The first step involves cell surface antibody staining for CD3, CD4 and CD25, followed by a second step in which populations of CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>intermediate</sup> and CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>high</sup> are divided for miRNA analysis and FoxP3 staining. The process of intercellular staining for FoxP3

## Chapter 2. Materials and Methods

requires cell fixing to maintain structural integrity followed by cell membrane permeabilization with saponin / detergent.

### 2.12.2 Cell surface staining

Lymphocytes from peripheral blood and mucosa were extracted using density centrifugation as outlined in sections 2.3.2 and 2.3.1 respectively.

For ex vivo surface staining, cells are suspended in 1 ml PBS in FACS tubes. Cells are counted and viability analysed by mixing 50  $\mu$ l Trypan blue with an equal volume of cell suspension, as outlined in section 2.3.3. The cell suspension is centrifuged at 1500 rpm and 4°C for 5 minutes, the supernatant is discarded and the pellet is re-suspended in 1 ml cold PBS and centrifuged at 1500 rpm and 4°C for 5 minutes. The supernatant is again discarded and the FACS tube agitated to re-suspend the pellet in the remaining PBS (approximately 100  $\mu$ l). Fluorochrome conjugated CD3, CD4 and CD25 are added, 2  $\mu$ l for every  $1.0 \times 10^6$  cells. Calibration samples are also required; a sample which has no antibodies added and separate samples each with a single antibody added.

All mixtures are protected from light and incubated at 4°C for 30 minutes. Mixtures are washed twice by adding 1 ml cold PBS and centrifuging at 1500 rpm and 4°C for 5 minutes, discarding supernatants between washes. Finally the FACS tube is agitated to re-suspend the pellet and PBS is added to make up to 300  $\mu$ l.

All cell suspensions are now ready for flow cytometry on the FACS ARIA following manufacturer's instructions.

### 2.12.3 Intracellular FoxP3 staining

The first step is to prepare the Permeabilization Buffer by diluting the 10x concentrate 1:10 with distilled water to create 4-5 mls of 1x working solution for each sample.

Centrifuge the sorted cells at 1500 rpm and 4°C for 5 minutes, discard the supernatant and agitate to dissociate the pellet. Fix the cells by adding 100  $\mu$ l of IC Fixation Buffer while vortexing the tube. Incubate at room temperature for 20 minutes in the dark then add 2 mls of 1x Permeabilization Buffer to each tube and

centrifuge at 1500 rpm and 18°C for 5 minutes. Discard the supernatant and repeat.

After discarding the supernatant, re-suspend the cells in 100 µL of 1x Permeabilization Buffer. Add 2 µl FITC conjugated FoxP3 and incubate in the dark at room temperature for 20 minutes.

When ready add 2 mls 1x Permeabilization Buffer and centrifuge at 1500 rpm and 18°C for 5 minutes, discard the supernatant and repeat with 2 mls PBS.

After discarding the supernatant, agitate the tube to dissociate the pellet and make up to 300 µl by adding approximately 200 µl PBS. Cell solution is now ready to proceed to FACS.

### 2.12.4 Data acquisition

Using the BD FACS Aria 1 flow cytometer, unstained and single colour control samples were used to calculate compensations. Based on forward and side scatter parameters a population of lymphocytes were first gated. CD3, CD4, CD25 and FOXP3 staining characteristics were analysed using BD FACSDiva™ software v6.0.

## 2.13 Statistical Analysis

Statistical analysis and graphs were created and presented using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA), available at [www.graphpad.com](http://www.graphpad.com). Tables were generated using Microsoft Office Excel 2007 software (Microsoft UK, Reading, UK).

All variables were considered to be non-parametrically distributed unless sample sizes were >20 and the results conformed visually to a Gaussian distribution. Comparisons were made between medians using the Wilcoxon Mann Whitney two sample rank sum test, otherwise known as the Mann Whitney U test (MW U). The results of the MW U test are displayed as shown in Figure 21, unless MW U values and n numbers are shown clearly in the accompanying illustrations, in which case statistical relevance is shown in the text using the p value only.

$$MW\ U = U\ statistic, n_1 = group\ 1, n_2 = group\ 2, p = p\ value$$

Figure 21. Mann Whitney U test. A non parametric hypothesis test for assessing whether one group of observations tend to have a value different to the other. The U statistic is a measure of the differences between the groups and defines if the null hypothesis can be rejected. The MW test generated two U values which vary between zero and  $(n_1 \times n_2)$ , it is usual for the smallest U value to be used. With group sizes <30 there is a difference between the groups if; it is obvious or there is a greater difference between U and  $\frac{1}{2}(n_1 \times n_2)$ .

Comparisons between groups in which  $n \leq 2$  was performed using the one tailed students t test. The comparison of the mean values for these groups using parametric tests is due to the inability of the statistical software to perform non parametric analyse between groups of such small n numbers.

The association between two variables is analysed using the Spearman's rank order correlation ( $r_s$ ) and is displayed as shown in Figure 22.

$$r_s\ (df) = r_s\ coefficient, p = p\ value$$

Figure 22. Spearman's rank order coefficient ( $r_s$ ). A measure of the association between two non parametrically distributed variables,  $r_s$  ranges between +1 and -1. The further from zero the value of  $r_s$ , the stronger is the association between the variables.  $P \leq 0.05$  rejects the hypothesis that the association happens by chance. Degree of freedom (df) = number of pairwise cases - number of groups.

The distribution of values within each group is presented as the inter quartile range (IQR) unless otherwise indicated.

For all analysis a p value  $\leq 0.05$  is considered statistically relevant. For consistency values are given to 3 decimal places.

## 2.14 Ethics

Human tissue and blood were obtained using protocols approved by the Southampton and South West Hampshire Regional Ethics Committee (REC) under the study titles;

1. "MiRNA coordination of inflammation-mediated changes in human gut epithelial tight junctions and innate immune cytokine expression". REC reference number 10/H0502/69.
2. "The role of proteases in gut inflammation". REC reference number 08/H0501/44.

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**Chapter 3. Developing a murine model of colitis to mimic the microRNA and cytokine profile in IBD**

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### 3.1 Chapter abstract

Murine models of colitis are widely used to study the expression and functional effects of miRNAs and inflammatory cytokines. MiR-155 has a prominent role in multiple inflammatory pathways, its dysregulation has been implicated in the aetiology of experimental colitis and its expression is increased in UC (Takagi et al., 2010, Van der Goten et al., 2012) and Crohn's (Fasseu et al., 2010). Experimental models have suggested an inverse relationship between the expression of miR-155 and miR-125 (Tili et al., 2007), a feature which drives an increased TNF- $\alpha$  expression, a pro-inflammatory cytokine that is thought to be central in the aetiology of IBD.

TNBS, Oxazolone and DSS are chemicals commonly used to induce murine colitis. The aim of this chapter is to develop a murine model of colitis that mimics the expression of miR-155, miR-125 and the cytokine profile of UC and Crohn's. Traditional theory holds that the pattern of cytokine expressions in Crohn's and UC favour a T<sub>H</sub>1 or T<sub>H</sub>2 profile respectively.

Mice were housed in accordance with local protocols and colitis induced with either TNBS, Oxazolone or DSS according to established protocols (Wirtz et al., 2007).

The expression of miR-155 is increased in variants of all 3 types of induced colitis. The expression of miR-155 is increased after administration of 3% TNBS, but not 2.5% or 2%, it is also increased in the 2% DSS model and increased on day 3 of the 2% Oxazolone model but not day 5. The expression of miR-125 is increased in the 3% TNBS model but not significantly altered in other models.

The largest increase in miR-155 expression was seen following rectal Oxazolone, in this model there was an increase in TNF- $\alpha$ , IL-10, IL-17. There was no change in INF- $\gamma$ , IL-4 or TGF- $\beta$ .

Changes in the expression of miR-155 are not consistent. Increases seen in the TNBS model at 3% may indicate that a threshold stimulus is required to trigger miR-155 expression. The increase seen at day 3 but not day 5 of the Oxazolone model suggests that the expression of miR-155 is an acute response that fades

## Chapter 2. Materials and Methods

over time. There was no significant change in the expression of miR-125. Cytokine profiles of all the experimental models did not favour either a T<sub>H</sub>1 or T<sub>H</sub>2 type response.

## 3.2 Chapter introduction

Human IBD provides a restrictive system for the experimental analysis of inflammatory pathways mainly due to the scarcity and biodiversity of samples. Murine models of colitis, on the other hand, provide a reproducible way to study the expression and functional effects of miRNAs and inflammatory cytokines. Because IBD does not exist in rodents, the first challenge is to create a murine model which mimics the histological appearance, disease distribution and cytokine expression profile of IBD closely enough to assume analogy between the model and human disease. The ideal murine model will express both miRNAs and inflammatory cytokines in proportion to those known in IBD.

Murine colitis can be induced using 3 main techniques; chemical induction, adoptive cell transfer and gene manipulation (Boismenu and Chen, 2000). The logistical ease of chemical induction makes this the most widely used model of experimental colitis. There are two main techniques; the first is through oral intake of dextran sulphate sodium (DSS) polymers which disrupt the integrity of the epithelial cell barrier and are directly toxic to epithelial cells. Over a prolonged period, usually a number of days, oral intake of DSS will induce an acute colitis characterised by bloody diarrhoea, ulcerations and mucosal infiltration with granulocytes. The second technique involves rectal administration of a haptening substance, such as 2,4,6-trinitrobenzene sulfonic acid (TNBS) or Oxazolone, both dissolved in ethanol. The ethanol serves two purposes, as a dissolvent and carrier for the TNBS or Oxazolone and to disrupt the mucus / epithelial cell barrier. Haptening substances are a useful method to study T helper cell dependent immune responses including cytokine secretion patterns, oral tolerance, cell adhesion and immunotherapy.

In experimental models the differential expression of miR-155 is strongly associated with the dysfunction of numerous pro- and anti-inflammatory mechanisms including  $T_{reg}$  function (Kohlhaas et al., 2009), IL-1 $\beta$  expression (Ceppi et al., 2009) and macrophage response (O'Connell et al., 2007). In UC, the expression of miR-155 is increased in the actively inflamed mucosa compared with healthy controls (Takagi et al., 2010), but decreased in IBD related dysplasia

### Chapter 3. Developing a murine model of colitis

compared with active UC (Olaru et al., 2011). The exact role of miR-155 in IBD is not known.

The expression of mir-125 in IBD is less certain. Many studies have not identified miR-125 as statistically deregulated (Takagi et al., Wu et al., 2008, Wu et al., 2010, Fasseu et al., 2010) although in experimental models using an LPS / TNF- $\alpha$  driven immune response it has been shown to be decreased and inversely related to miR-155 (Tili et al., 2007).

Ulcerative colitis is considered to be a T<sub>H</sub>2 and Crohn's a T<sub>H</sub>1 mediated inflammatory disease, although this assertion is losing favour as discussed previously. However in experimental models it is still an important assumption that those models in which the cytokine milieu is balanced in favour T<sub>H</sub>2 (IL-4, IL-5 and IL-13) are mimicking UC and those in which the cytokine milieu is balanced in favour T<sub>H</sub>1 (INF- $\gamma$ , IL-2 and TNF- $\alpha$ ) are mimicking Crohn's. Despite the potential pitfalls of these assumptions, murine models offer a useful method of measuring miRNA and inflammatory cytokine levels under conditions which can be mapped into human disease and inflammatory processes.

In this chapter a variety of murine models are analysed to determine if they mimic either UC or Crohn's as measured by the miRNA and cytokine expression profiles. The use of 3 different chemical induction agents are used as previously described, TNBS, Oxazolone and DSS with the exact timing of induction and sample harvest outlined in section 2.8.

A number of cytokine profiles are described, they fall into a number of categories; those which favour a T<sub>H</sub>1 type response (TNF- $\alpha$  and INF- $\gamma$ ), those which favour a T<sub>H</sub>2 type response (IL-4 and IL-10), T<sub>H</sub>17 function (IL-17) and other genes of interest including those involved in T<sub>reg</sub> function (FOXP3, TGF- $\beta$  and IL-10).

The hypothesis is that TNBS, Oxazolone or DSS can be used to induce colitis in mice in which the pattern of miRNA and cytokine expression mimics that of IBD. It is expected that the colonic mucosal expression of miR-155 is increased and the expression of miR-125 is decreased.

### 3.3 Determining colitis severity

In all of the experimental models the mice developed significant weight loss with a majority of the mice reaching a nadir of -15% weight change between days 2 and day 4. In all models the weight of each mouse was significantly reduced from base line at the time of harvesting.

#### 3.3.1 The 2% Oxazolone model

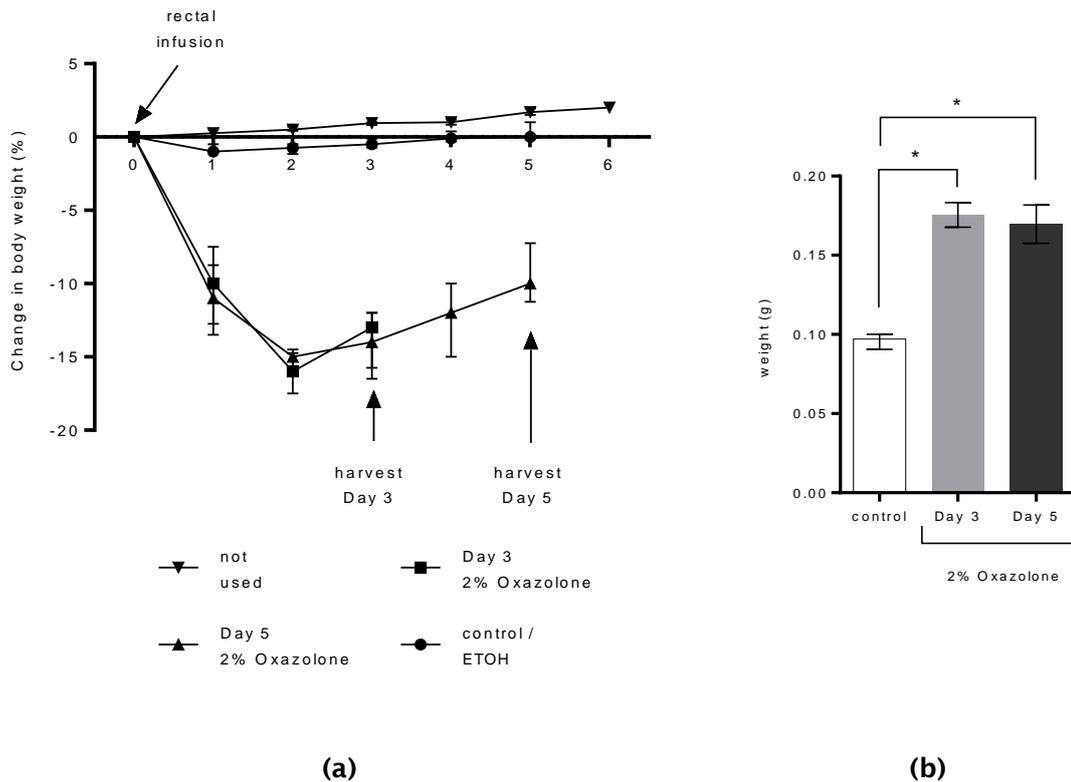


Figure 23. Determining inflammatory activity by weight change and colon weight in the 2% Oxazolone model. Inflammatory activity was measured in each experimental model by analysing daily weights, and by measuring the weight of the distal 4cm of colon after removal of faeces. (a) The daily % weight change for each group of mice, control group (n = 8), day 3 harvested mice (n = 6), day 5 harvested mice (n = 6) and mice kept under the same laboratory conditions (n = 6). (b) The weight of the distal 4cm of resected colon . Median values and IQR are shown. Medians compared with the two tailed Mann Whitney U test \* p < 0.05.

The example shown in Figure 23a is the daily weight change in the 2% Oxazolone model of colitis. In this model the nadir of -16.0% (IQR -14.0% – -18.0%) weight change occurs on day 2. The significant weight change is still present when

### Chapter 3. Developing a murine model of colitis

harvested on day 3 and on day 5, -13.0% (IQR -12.0% - -18.0%) and -10% (IQR -5.0% - -12.0%) respectively.

In the 2% Oxazolone model the weight of the distal 4 cm of colon is significantly greater in the day 3 and day 5 models compared with controls, Figure 23b. At day 3 the colon weighs 0.177 g (IQR 0.167 - 0.182,  $p < 0.001$ ) and at day 5 the colon weighs 0.170 g (IQR 0.159 - 0.176,  $p < 0.001$ ).

Visual inspection of the resected colons show the presence of thickening, discrete strictures, dilated regions of colon and soft stools when compared with controls.

#### 3.3.2 The TNBS model

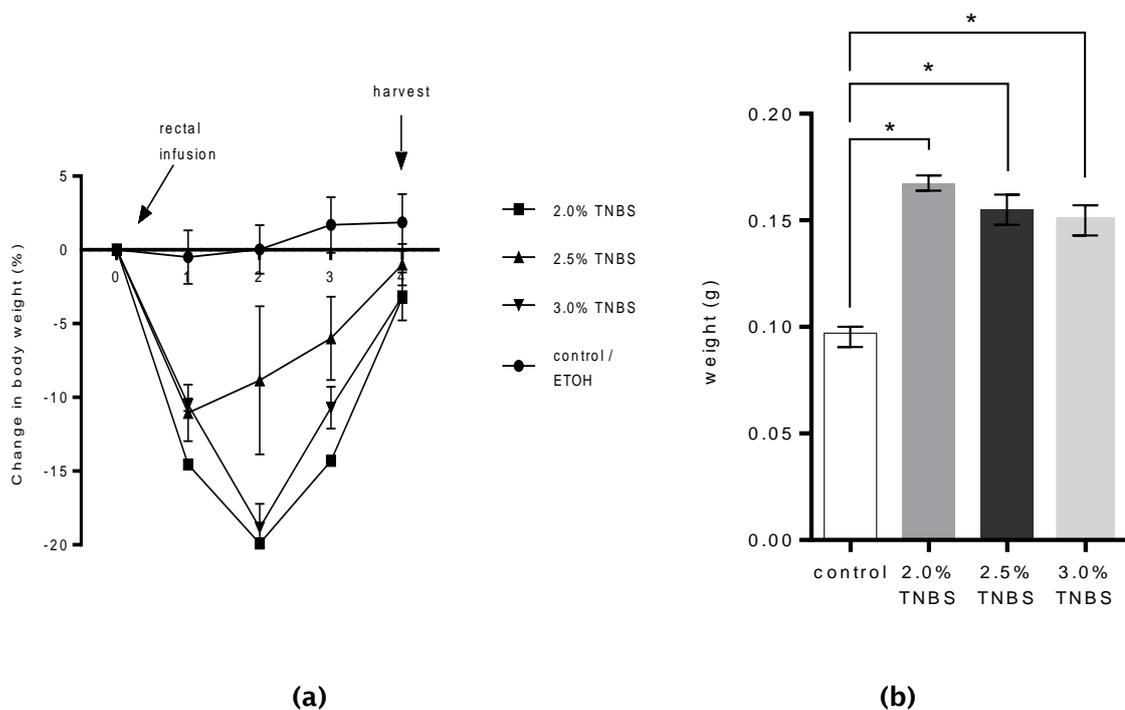


Figure 24. Determining inflammatory activity by weight change and colon weight in the TNBS model. Colons are harvested 5 days after rectal infusion of 2.0% (n = 2), 2.5% (n = 2) and 3.0% (n = 2) TNBS and compared with controls (n = 8). (a) Inflammatory activity was measured in each experimental model by analysing daily weights, and by (b) measuring the weight of the distal 4 cm of colon after removal of faeces. Mean values are compared with the students t test. Values shown are the means and ranges, \*  $p < 0.05$

The greatest weight change is measured 2 days after TNBS infusion and is greatest in the 2.0% TNBS model, 19.9% (range 19.8 – 20.0), Figure 24. In all 3 variations of the TNBS model the colon weight on the day of harvest is significantly greater than in the control group.

Visual inspection of the resected colons and faeces show that in all of the TNBS treated mice the faeces was looser than in the controls. In one of the 2.5% and one of the 3.0% colons there were obvious strictures and increase stiffness. The remaining colons looked macroscopically normal.

### 3.3.3 The DSS model

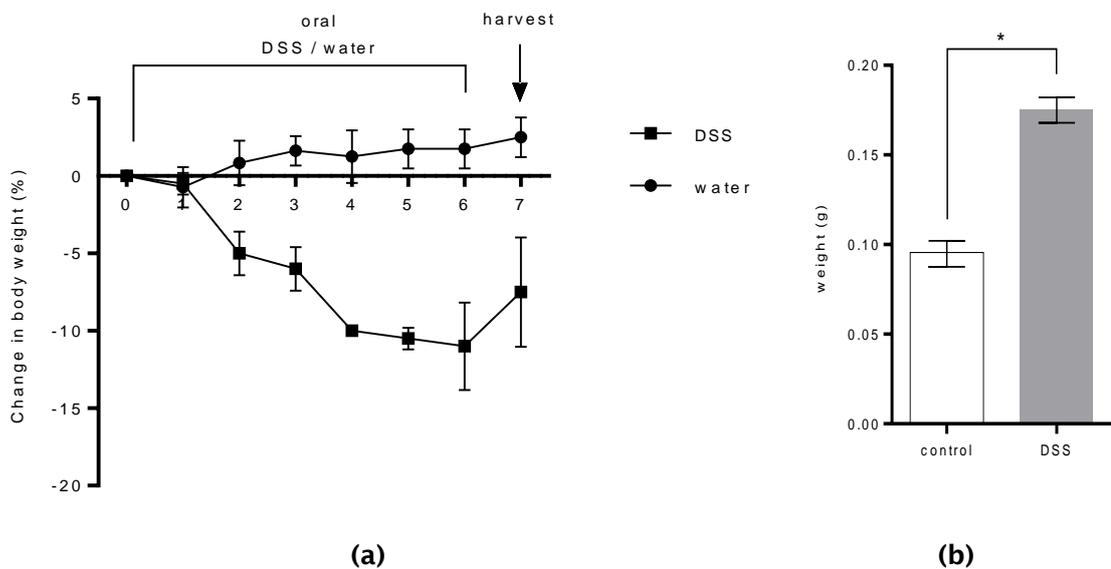


Figure 25. Determining inflammatory activity by weight change and colon weight in the DSS model. Colons are harvested on day 7 after a 6 day course of DSS (n = 2) and from controls (n = 4). (a) Inflammatory activity was measured in each experimental model by analysing daily weights, and by (b) measuring the weight of the distal 4 cm of colon after removal of faeces. Mean values are compared with the students t test. Values shown are the means and ranges, \* p < 0.05

### Chapter 3. Developing a murine model of colitis

The greatest weight change is measured on day 6, -10.0% (range -10.0 - -11.0), although chronic administration of DSS results in a change in weight which is less aggressive than that seen in TNBS or Oxazolone, and due to the duration of administration appears to have a more prolonged effect on body weight, Figure 25. On day 7 the mean change in body weight is -7.5% (range -5.0 - -10.0).

There was no macroscopic difference in the appearance of the colons after resection between the DSS treated mice and controls although the stools were looser in the treatment group.

The weight of the distal 4 cm of colon is significantly greater in the DSS group, 0.175 g (range 0.170 - 0.180) compared with control, 0.095 g (range 0.086 - 0.1030),  $p < 0.001$ .

### 3.4 The expression of miR-155 and miR-125 in murine models of colitis

#### 3.4.1 The expression of miR-155 is raised following rectal administration of 3.0% TNBS but not 2.5% or 2.0% TNBS

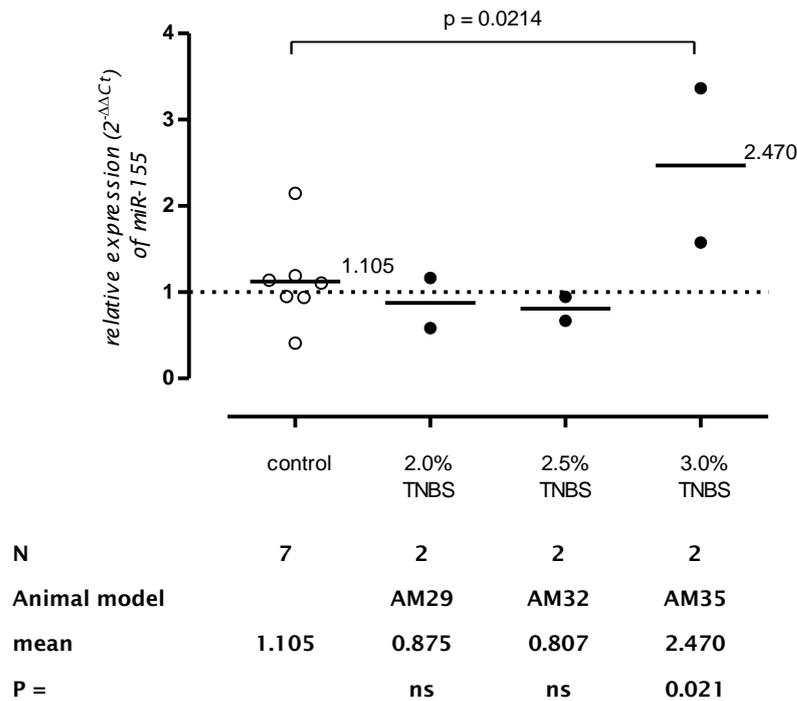
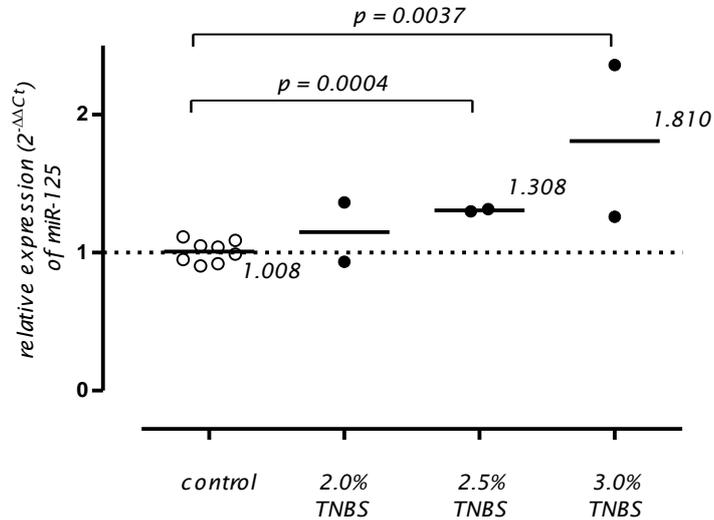


Figure 26. The expression of miR-155 in a TNBS model of murine colitis. The expression of miR-155 was normalised to sno202 and compared against control (50% ethanol infusion). Comparisons of the means were calculated using the one-tailed unpaired students t test.

The relative expression of miR-155 in the colonic mucosa of mice who received a rectal infusion of 3.0% TNBS is significantly increased by 2.470 fold ( $\pm 1.267$  s.d.,  $p = 0.021$ ) compared with control. There was no significant difference in the expression of miR-155 in identical murine models AM29 and AM32 in which 2.0% and 2.5% TNBS were infused respectively

**3.4.2 The expression of miR-125 is raised following rectal administration of 3.0% TNBS but not 2.5% or 2.0% TNBS.**



	control	2.0% TNBS	2.5% TNBS	3.0% TNBS
N	8	2	2	2
Animal model		AM29	AM32	AM35
Mean	1.008	1.150	1.308	1.810
P =		ns	ns	0.021

Figure 27. The expression of miR-125 in a TNBS model of murine colitis. The expression of miR-125 was normalised to sno202 and compared against control (50% ethanol infusion). Comparisons of the means were calculated using the one-tailed unpaired students t test.

The relative expression of miR-125 is significantly increased in both the 2.5% and 3.0% TNBS models, but not the 2.0% model when compared against control. In the 2.5% TNBS model the relative expression of miR-125 is increased by 1.308 fold ( $\pm 0.011$  s.d.,  $p < 0.001$ ), and in the 3.0% TNBS model the relative expression of miR-125 is increased by 1.810 fold ( $\pm 0.778$  s.d.,  $p = 0.004$ ). There appears to be an increasing expression of miR-125 with increasing concentration of TNBS suggesting a dose dependent response, although this is not statistically significant,  $r_s(2) = 1.000$ ,  $p = 0.083$ .

**3.4.3 The expressions of miR-125 and miR-155 are increased following oral administration of 2% DSS.**

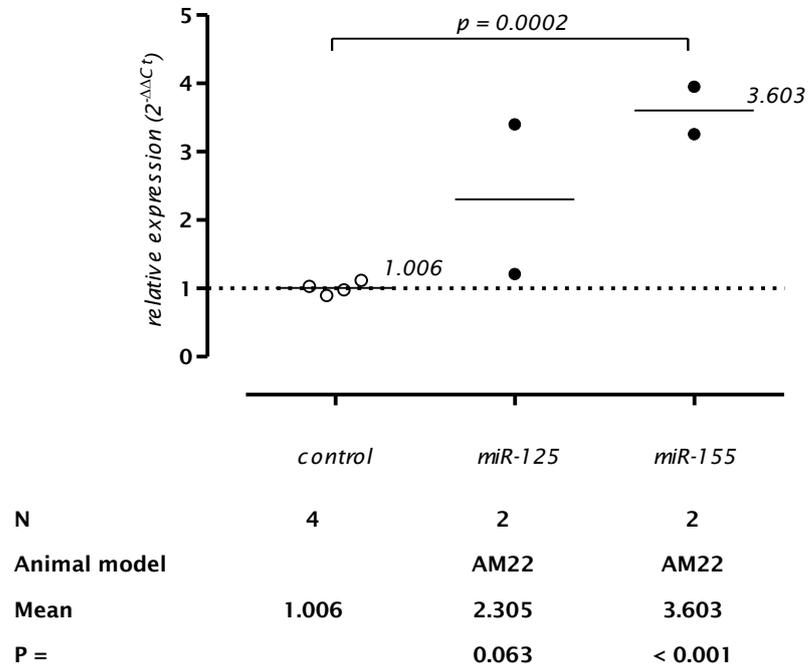
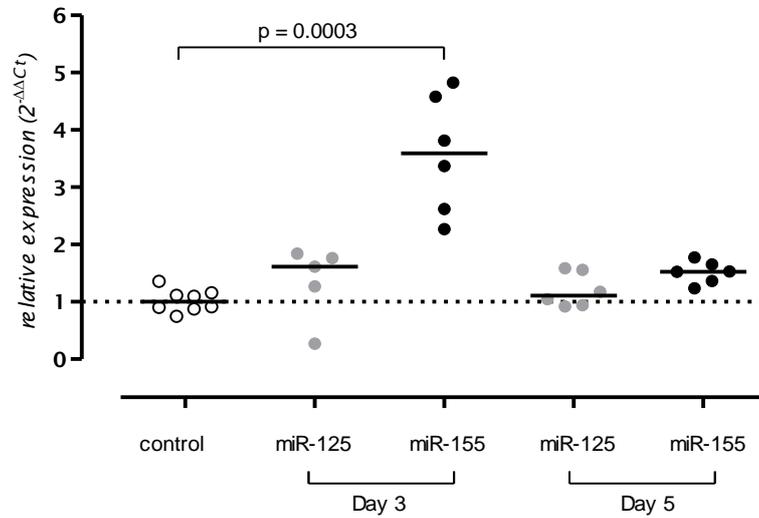


Figure 28. The expression of miR-125 and miR-155 in a 2% DSS model of colitis. The expressions of miR-125 and miR-155 were normalised to sno202 and compared against control (oral administration of water). Comparisons of the mean values were calculated using the one-tailed unpaired students t test.

The expressions of miR-125 and miR-155 are increased in the colonic mucosa following the oral administration of 2% DSS compared with control. The expression of miR-155 is significantly increased by 3.603 fold ( $\pm 0.491$  s.d.,  $p < 0.001$ ), and the expression of miR-125 is increased by 2.305 fold but not significantly ( $\pm 1.549$  s.d.,  $p = 0.063$ ).

### 3.4.4 The expression of miR-155 is increased on day 3 and day 5 following rectal 2% Oxazolone.



N	8	5	6	6	6
MW U (compared with control)		9	0	13	1
Animal model		AM24	AM24	AM24	AM24
Median	1.005	1.615	3.593	1.110	1.528
IQR	0.882 - 1.149	0.770 - 1.803	2.533 - 4.641	0.935 - 1.566	1.333 - 1.689
P =		0.064	< 0.001	0.091	< 0.001

Figure 29. The expression of miR-125 and miR-155 in a 2% Oxazolone model of colitis at day 3 and day 5. The expressions of miR-125 and miR-155 were normalised to sno202 and medians compared with the one tailed Mann Whitney U test.

The expression of miR-155 is increased on day 3 and day 5 compared with control. On day 3 the expression of miR-155 is increased by 3.593 fold (IQR = 2.533 - 4.641,  $p < 0.001$ ), and on day 5 the expression of miR-155 is increased by 1.528 fold (IQR = 1.333 - 1.689,  $p < 0.001$ ).

There is no significant change in the expressions of miR-125 on either day 3 or day 5.

## 3.5 The expression of inflammatory cytokines in murine models of colitis

The hypothesis is that TNBS, Oxazolone or DSS can be used to induce colitis in mice and this inflammation mimics the pattern of cytokine mRNA expression in IBD. It is expected that the different models of colitis will favour either a T<sub>H</sub>1 or T<sub>H</sub>2 type inflammatory cytokine profile and thus provide a model in which it is possible to analyse the influence of miRNA expression on these cytokines.

The aim of this section is to describe the T<sub>H</sub>1 (TNF- $\alpha$  and INF- $\gamma$ ), T<sub>H</sub>2 (IL-4 and IL-10), and cytokines of interest (FOXP3, TGF- $\beta$  and IL-17) expression profiles in the Oxazolone and TNBS models of colitis.

### 3.5.1 Cytokine expression following rectal administration of 2% Oxazolone

Cytokines were measured on day 3 (n = 3) and day 5 (n = 4) following rectal administration of 2% Oxazolone and compared against controls (n = 2). The expression of various cytokine mRNA expressions were analysed by SYBR<sup>®</sup> Green RT-qPCR after RNA extraction from murine colonic resection samples following the RNeasy™ protocol outlined in section 2.4.2.

There is no statistically significant differential expression of TNF- $\alpha$ , INF- $\gamma$ , IL-4, IL-10, TGF- $\beta$ , or FOXP3 in the colonic mucosa using this model of murine colitis. The expression of TNF- $\alpha$  appeared to be increased on day 3 following 2% Oxazolone administration but this did not reach significance.

Following the rectal administration of 2% Oxazolone the relative expression of IL-17 mRNA in the murine colonic mucosa is 3.249 fold ( $\pm$  0.359 s.d., p = 0.007) when compared with control but is not altered on day 5, 1.143 fold.

During the analysis of IL-4, IL-17 and FOXP3 on day 3 and INF- $\gamma$  on day 5 one of the samples failed to amplify during the PCR resulting in fewer results in these experiments. The results shown in Figure 30, take these reduced numbers into account.

### Chapter 3. Developing a murine model of colitis

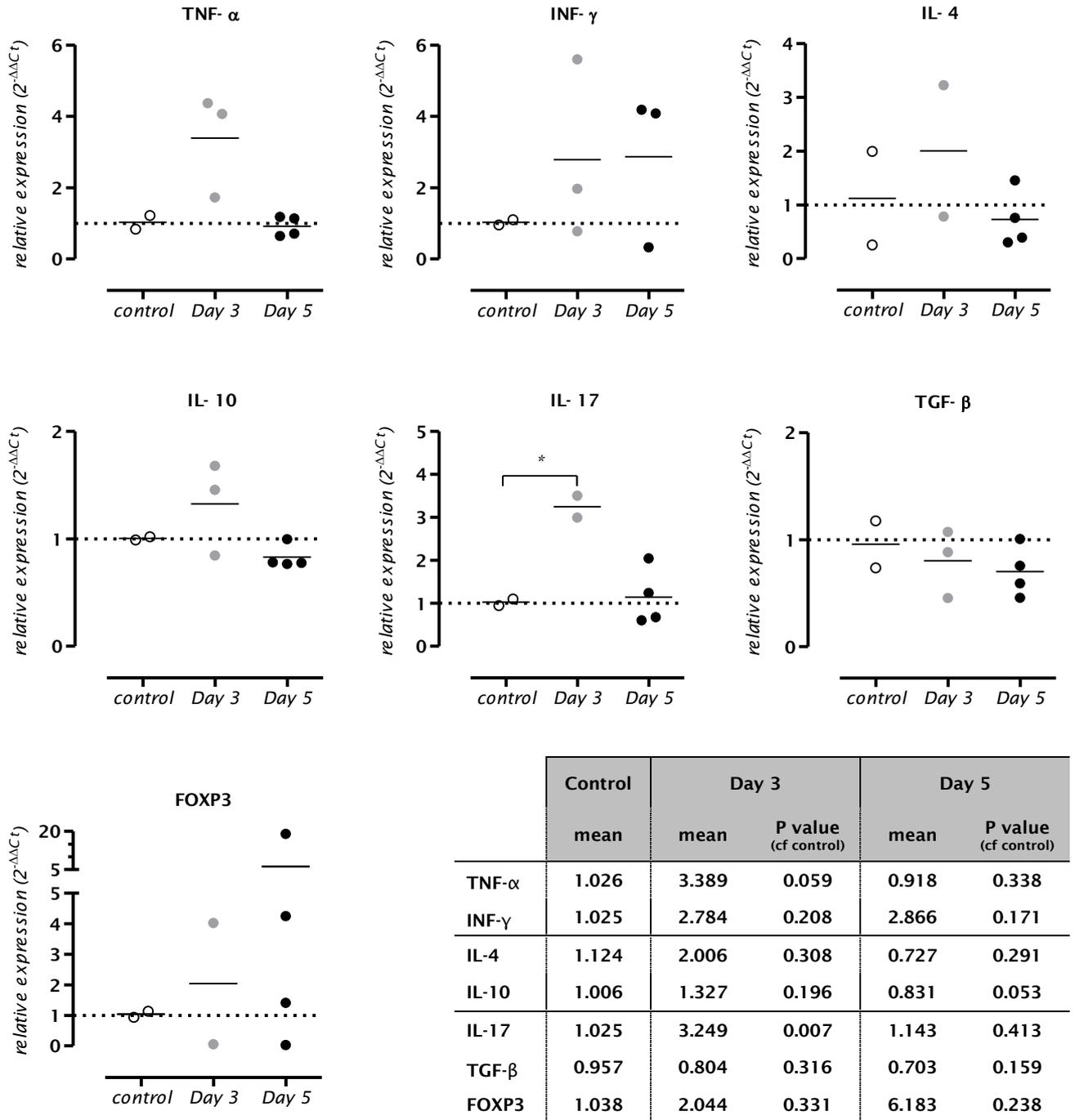


Figure 30. The expression of cytokine mRNA in murine colonic mucosa following administration of 2% Oxazolone. The cytokine expressions were analysed by RT-qPCR, normalised to  $\beta$ -actin and compared against controls. Comparisons of the means were performed using the one tailed students t test. (\*  $p \leq 0.05$ ).

## 3.6 Chapter discussion

Little is known about the mechanisms which predispose, trigger or drive IBD. In the middle of the 20<sup>th</sup> century the leading ideas revolved around an infectious aetiology although the inability to find a causative agent and the effectiveness of steroid therapy has moved the prevailing ideas towards thinking of IBD as an immunological disease. Murine models play a crucial role in studying and advancing our understanding of immunological mechanisms because of our limited ability to interrogate human disease. Since the 1980s there has been an explosion in the number of experimental models of colitis. There are now almost limitless models available providing a wide range of pathological, phenotypic and / or immunological characteristics (Boismenu and Chen, 2000, Strober et al., 2002).

The ideal murine model of colitis would reflect all characteristics of IBD. The ideal murine model would have the same phenotypic presentation, disease distribution, histological changes response to the microbiome, cellular immune mechanisms, cytokine profile and response to gene dysfunction as that seen in IBD. One of the many challenges for murine models of colitis is that although IBD or diseases very similar exist in cats, dogs and higher order mammals, it is not known to exist in mice, and currently available experimentally induced murine models do not mimic all aspects of IBD in one. Experimental models therefore are chosen depending on what aspect or mechanism of colitis is being studied.

The aim of this chapter is to develop a model of colitis in which the miRNA and cytokine profiles match those that are seen in either UC or Crohn's with a view to investigating the effect of manipulation of miRNA levels on cytokine profiles. The logistical ease of chemical induction makes this the most widely used method of experimentally induced colitis. Three chemical induction models are used separately; oral DSS, rectal TNBS and rectal Oxazolone. Variations within these models using different doses and time points were used to optimise responses.

In 2009 there were approximately 1000 known miRNAs, Figure 17, of which miR-155 was arguably attracting most interest due to its increasing prominence as a factor in numerous inflammatory pathways (Teng and Papavasiliou, 2009) and diseases (Faraoni et al., 2009), it was and still is generally accepted to have an

### Chapter 3. Developing a murine model of colitis

abnormal expression and important role in the aetiology of IBD. Preliminary data produced by our laboratory had shown that miR-155 has an increased expression in resected human Crohn's and UC, appendix 8.1.1<sup>9</sup>, and also an increased expression in TNBS induced mouse colitis, appendix 8.1.2<sup>10</sup>. At the same time interest in the control of TNF- $\alpha$  by the influence of miR-155 and miR-125 was coming to the fore (Tili et al., 2007, O'Connell et al., 2007). It was therefore decided to investigate the expression of miR-125 and miR-155 further.

Despite the preliminary results and the previously published literature, this study revealed an inconsistent expression of miR-155 across the different models and their variants. One feature of the inconsistency is demonstrated by the TNBS model in which there is no increase in miR-155 in the 2% and 2.5% models, but a statistically significant increase in the 3% model. This would suggest that there is a threshold concentration of TNBS that is required to stimulate miR-155 expression. Since the carrier substance, ethanol is unchanged between the groups, the difference in miR-155 expression may be explained by increased antigen presentation to the mucosal immune system by increased amounts of TNBS.

A feature of the Oxazolone model is the increased miR-155 at day 3 but not day 5. This is consistent with previous studies that have found the expression of miR-155 to be maximal between 12 and 24 hours after chemical induced colitis (Ceppi et al., 2009, O'Connell et al., 2007), with other inflammatory cytokines settling to normal over 4 to 5 days. This feature is mirrored in this Oxazolone model in which the weight change is beginning to recover after the nadir on day 3.

There is a significant increase in the expression of miR-155 in the DSS model. The expression of miR-125 mirrors that of miR-155 in the TNBS and DSS models, but does not show a change in expression in the Oxazolone models contrary to the expected opposing changes in miR-155 and miR-125 recently reported (Tili et al., 2007). A closer examination of the literature reveals that the expression of miR-155 and miR-125 is less clear than is often quoted.

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<sup>9</sup> Unpublished results, experiments performed by Dr Rebecca Morgan-Walsh.

<sup>10</sup> Unpublished results, experiments performed with Dr Elena Vorobeva

The primary studies associating miR-155 with IBD utilise either murine or in vitro experimental models, however, there is little in the way of direct evidence for the differential expression of miR-155 in IBD. The expression of miR-155 has been reported as increased in the colonic mucosa by Takagi et al., (2010) and by Van der Goten et al. (2012) in an abstract presented at the 2012 European Crohn's and Colitis Organisation annual conference. Although in the earlier study the control group were not healthy individuals as stated, but were patients undergoing colorectal cancer resection and the relative increase in miR-155 was not measured against a control group, but a single individual result. We know that patients with colorectal dysplasia and cancer (Olaru et al., 2011) have differential expressions of certain miRNAs and so comparing miRNA profiles against these cannot be considered a "healthy control".

Neither Wu et al. (2008) or Fasseu et al. (2010) found an altered expression of miR-155 between active UC and healthy mucosa, although under-reporting may be a feature of the study by Fasseu et al. (2010) due to the stringent 5 fold change in expression criteria required for miRNA expression to be considered significant. It is already well established that much smaller fold differences for many miRNAs result in important biological differences. For Crohn's, despite Fasseu et al. (2010) reporting an increase in the miR-155 expression in active and inactive colonic Crohn's compared with healthy mucosa Wu et al. (2010) did not show a differential expression in either terminal ileal or colonic Crohn's. In later chapters the focus will be on 7 "candidate" miRNAs shown by microarray and RT-qPCR to have consistently deranged expression in active UC compared with healthy control, miR-155 and miR-125 do not feature amongst them, however in the microarrays, the expression of miR-155 is shown to be 2.74 and 1.64 fold increased in active mucosal UC and Crohn's respectively. These results are displayed in section 8.3.

Taken together the expression of miR-155 is raised in UC and Crohn's and using this as a surrogate marker of miRNA expression as a whole, a model developed to reflect miRNA activity in colitis should also have an increased expression of miR-155. The expression of miR-125 is less well understood. MiR-125 targets TNF- $\alpha$  and is shown to be decreased in circumstances in which TNF- $\alpha$  is increased (Tili et al., 2007). The lack of differential expression of miR-125 between UC and healthy

### Chapter 3. Developing a murine model of colitis

mucosa, but the increased expression in experimental models focusing on the macrophage response to LPS / PAMPs would suggest that the inverse relationship between miR-155 and miR-125 is a cell specific phenomenon, which may explain the findings shown in this chapter.

Ulcerative colitis is considered to be a  $T_H2$  and Crohn's a  $T_H1$  mediated inflammatory disease respectively, although this assertion is losing favour due to the discovery of other important T helper cell subsets such as  $T_{regs}$  and  $T_H17$  cells and the successful use of TNF- $\alpha$  inhibitors for UC in clinical practice. None the less it is often helpful to achieve a polarised  $T_H1$  (INF- $\gamma$ , IL-2 and TNF- $\alpha$ ) or  $T_H2$  (IL-4, IL-5 and IL-13) inflammatory response in murine models to analyse specific immune mechanisms.

Following the results of miR-155 expression it was decided to take the Oxazolone model forward to analyse cytokine profiles. The decision was made on the basis that Oxazolone is thought to mimic mostly the  $T_H2$  inflammatory response and so be representative of UC (Strober et al., 2002, Boismenu and Chen, 2000). The increased expression of miR-155 at day 3 but not day 5 if reflected in a differential cytokine expression may also point to an association between miRNA and cytokine expression.

There were no significant changes in the expressions of TNF- $\alpha$ , INF- $\gamma$ , IL-4, IL-10, TGF- $\beta$  or FOXP3 in the Oxazolone model. The cytokine response to Oxazolone administration is non-specific in line with previous authors who have also found a non specific inflammatory response when measured histologically or with serum cytokines (Celinski et al., 2010).

An interesting finding is the significant increase in IL-17 at day 3 but not day 5 which mirrors the changes seen in miR-155 in the Oxazolone model and the increase seen in both IL-17 (Fujino et al., 2003) and miR-155 in active UC. This suggests a  $T_H17$  response at day 3 that has decreased by day 5, although the picture is not entirely clear as the expression of IL-17 is driven in part by changes in TGF- $\beta$ , as well as other cytokines including IL-6, IL-23 and IL-1 $\beta$  (Cosmi et al., 2008)

The major drawback for murine models is that they often fail to mimic either a  $T_H1$  or  $T_H2$  inflammatory phenotype, but result in a non-specific inflammatory response (Celinski et al., 2010). The reasons for this are multifactorial but revolve around precise experimental conditions. Dextran sulphate sodium (DSS) polymers are directly toxic to epithelial cells and disrupt the integrity of the epithelial cell barrier. Taken over a number of days, DSS induces an acute colitis characterised by bloody diarrhoea, ulcerations and mucosal infiltration with granulocytes. The primary immunological response to DSS involves activation of the macrophage response and pro-inflammatory cytokines including a large increase in  $TNF-\alpha$ , and is therefore thought of primarily as a  $T_H1$  model in which the adaptive immune response does not play a major part (Wirtz and Neurath, 2007). The DSS model is therefore useful to study the innate immune mechanisms and epithelial repair. However, in a review of murine models of colitis by Strober et al. (2002), it is explained that depending on the conditions, DSS can also stimulate a secondary T cell response leading to either a  $T_H1$ ,  $T_H2$  or mixed helper cell response.

The GI tract lumen is packed full of potentially immunogenic antigens, which under normal circumstances do not cause an immunological response. The ethanol in the TNBS and Oxazolone models disrupts the epithelial barrier and the TNBS or Oxazolone binds to the normally innocuous antigens (haptens). Thus the haptens are presented to the immune system by TNBS or Oxazolone through a disrupted epithelia barrier. Haptenising substances such as TNBS and Oxazolone are a useful method to study T helper cell dependent immune responses including cytokine secretion patterns, oral tolerance, cell adhesion and immunotherapy. The model used is determined by which part of the immune response is being studied. Often small changes in experimental design lead to changes in the immune response, including the degree of exposure to each chemical, genetic variations or even the use of a pre-sensitisation step. The method used will also depend on how you want to measure the inflammatory response, i.e. via histology, disease distribution or cytokine expression. When measuring cytokines the TNBS models favour a  $T_H1$  type response, where as other haptenising chemicals such as Oxazolone favour a  $T_H2$  type response with a marked increase in  $IFN-\gamma$ , IL-4 and IL-5. The inflammatory

### Chapter 3. Developing a murine model of colitis

reaction caused by Oxazolone lasts 4 to 5 days which is less than TNBS suggesting TNBS induces a more chronic inflammatory phenotype (Wirtz and Neurath, 2007).

In summary, although a number of the variations within each model presented in this chapter resulted in an increased expression of miR-155, this did not result in the reciprocal decrease in the expression of miR-125. Also, none of the murine models analysed here demonstrated a cytokine expression profile consistent with either a  $T_H1$  or  $T_H2$  response. The inconsistencies shown here are mirrored in the literature (Wirtz and Neurath, 2007), although there are a number of limitations within the experimental design which need to be taken into account when interpreting the results. A major obstacle to interpretation is the variability of the results within certain groups. Variability occurs as a result of two factors; technical variability and biological variability. Throughout these experiments there are several procedures which minimise technical variability including normalising gene expression to an endogenously expressed gene and performing individual samples in duplicate. The second factor is biological variability which is a recognised feature of miRNAs (Mestdagh et al., 2009) and cytokines. To overcome biological variability a large sample number is often required, however in some of the experiments reported in this chapter the sample sizes were small,  $n = 2$ . It is therefore difficult to draw meaningful conclusions from some of the experiments. However the purpose of these preliminary experiments was to find if there was a consistent and clear experimental design which would produce an appreciable increase in miR-155 alongside a polarised  $T_H1$  or  $T_H2$  cytokine response. Additional mice could have been utilised however, the fundamental ethical principles underpinning the mice experiments require using a few mice as possible to achieve the purpose of the study (University of Southampton, 2011). Experiments were drawn to a close with no additional mice due to inconsistent results and a positive move towards human tissue to investigate miRNA profiles in IBD.

Mice were administered 3 different types of chemical induction and their colons were harvested at defined time points, Table 24, in line with published protocols (Wirtz et al., 2007). Methods of assessing inflammatory activity at the time of harvesting include histological, high resolution microscopic endoscopy, body weight, colon weight or stool observations (Wirtz et al., 2007). As a primary

measure of the effect of the induction agent and measure of inflammatory activity the body weight and distal 4 cm of colon weight are shown in Figure 23 to Figure 25. For each of the models there is a decrease in total body weight and an increase in colon weight, also there were visual features and appearances of the faeces consistent with active colitis. No histology is shown to directly confirm that each model had developed colitis, which is a limitation of this chapter. However, these previous features show that each model was harvested at a clinically appropriate time and were suffering a clinically significant degree of inflammation.

The limitations discussed above would suggest that in hindsight an alternative approach may have strengthened the study design. Confirmation of inflammation at harvesting by histology and / or using a scoring algorithm (Shinzaki et al., 2012, Dohi et al., 2005) would directly confirm colitis and perhaps help reduce biological variance by dividing the effect of chemical induction by severity. Rather than focusing on miR-155 and miR-125 specifically from the outset, performing microarrays may have exposed additional miRNAs of interest. The final important alteration is the number of mice used. The number used is finely balanced between requiring a large enough number to reach meaningful significance and avoiding outliers of biological variance, and adhering to ethical principles. Defining a larger number in each group would have given more weight to the results presented.

When interpreting experimental models generally, there are certain considerations which must be taken into account. The first is the difference between induced colitis in mice and IBD in humans, the second is the difference in miRNA expression and function between species, and the third is the difference in cytokine expression and function between species. All three factors can make translation of murine models to human biology challenging and study results misleading.

Another obstacle to correct interpretation of miRNA results generated by experimental colitis is the assumption that miRNA which are named numerically similar between species are identical. MicroRNA nomenclature indicates an orthology but conveys no information about expression and function (Ambros et

### Chapter 3. Developing a murine model of colitis

al., 2003). So although similarly named miRNAs across species are supposed to have an identical nucleotide sequence, they do not all have, and although conservation of genes between species is meant to indicate an evolutionary or survival advantage for possession of that gene, a similar function across species is again an assumption.

The further from human disease experiments are performed, the less they can be interpreted as significant for that human disease, To define the miRNA profile of IBD more accurately, the next step is to perform miRNA profiles on human colonic tissue with a view to identifying which miRNAs are most deregulated in active disease

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**Chapter 4. The microRNA signature of IBD  
and bioinformatic search for potential  
targets**

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## 4.1 Chapter abstract

There have been in excess of 2000 human miRNAs identified to date, of which over 70 have been associated with IBD. The functional role of miRNAs in IBD remains elusive, but with over 50% of protein coding genes predicted to fall under the influence of miRNAs, and experimental models that show a crucial role for miRNAs in many inflammatory pathways, it stands to reason that miRNAs play an important part in the aetiology of IBD.

Previous studies have identified miRNA signatures for UC, but the results have been inconsistent and difficult to interpret. Part of the challenge of existing studies is their lack of concordance; some reasons for this are the grouping of patients with clinically important differences including differing phenotypes and patients taking a variety of immune altering treatments. In this study a distinct group of miRNAs are shown to be deregulated in the actively inflamed sigmoid mucosa of a homogeneous group of treatment naive patients. Potential targets of these miRNAs are identified.

Using microarray, seven candidate miRNAs are identified to be consistently deregulated in mucosal biopsies from active UC. The relative expression compared with healthy control is analysed by RT-qPCR. The expressions of miR-31, -146b and -223 are increased by 10.45, 2.26 and 3.45 fold respectively and the expressions of miR-194, -200b, -375 and -422a are decreased by 0.39, 0.46, 0.28 and 0.26 fold respectively.

Subgroup analysis reveals that the expression of miR-31 but not miR-375 is lower in patients with both active UC and active Crohn's who are taking Azathioprine alone compared with patients taking 5-ASA alone, this suggests that combining patients taking different medication regimes can affect the miRNA signature of that group of patients.

Using in silico analysis >3000 potential mRNA are identified. Thymic Stromal Lymphopoietin (TSLP) is identified as a target for 4 of the 7 candidate miRNAs.

## 4.2 Chapter introduction

To date there are 2042 human miRNAs identified of which 72 have been shown to be either increased or decreased in the mucosa of active IBD compared with healthy mucosa (Wu et al., 2008, Wu et al., 2010, Fasseu et al., 2010, Van der Goten et al., 2012). Of the 72 deregulated miRNAs found between the four published microarray studies of IBD there are 65 that are deregulated in UC, however there are only 10 that are identified in 2 or more of the studies. The poor concordance between studies may reflect group heterogeneity or pre-existing immunotherapy, both of which will impact on the interpretation of the results and their clinical usefulness.

To overcome the pitfalls of previous studies and to ensure the results reflect a clinically applicable disease group there are a number of stringent sample inclusion and exclusion criteria have been used. The first is the distinction that is made between patients attending endoscopy and those undergoing resection. Clinically these two groups represent entirely separate phenotypes with different long term outcomes and potentially different aetiological factors. Samples are therefore only collected from patients attending endoscopy. The second is sample collection from a specific region of the colon. There is debate over regional differences in miRNA expression, so to exclude this factor only sigmoid biopsies are included. The third inclusion criteria is that patients should be free from the immune altering influence of medication, as it stands to reason that medications which influence immune function may influence miRNA expression.

Using microarray and validation of the results with RT-qPCR, the expressions of 377 of the most prominent miRNAs are investigated primarily in active sigmoid UC, and for comparison in active sigmoid Crohn's. Firstly the microarray quantifies the relative expression of each of the 377 miRNAs in active UC compared with a healthy control; this allows the miRNAs to be ranked in order of relative expression for each microarray. Previous studies have suggested that about 5% of miRNAs have an expression that is differentially expressed by  $\pm 2$  fold compared with healthy controls (Wu et al., 2008). Therefore, concentrating on the 20 miRNAs which have the greatest increase in expression and those 20 miRNAs with the

greatest decrease in expression for each microarray reveals that seven “candidate miRNAs” are consistently differentiated in UC.

Following the identification of 7 differentially expressed miRNAs in active UC, in silico analysis using several freely available but entirely different target gene prediction algorithms are used to predict potential mRNA targets.

### 4.3 The search for differentially expressed microRNA in sigmoid UC by microarray

The initial search for differentially expressed miRNAs involves the quantitative analysis of many hundreds of miRNAs from each sample using microarray. The patient characteristics of microarray experiments conducted in UC and Crohn's are shown in Table 26 and Table 27 respectively.

		Control (n = 6)	Active UC (n = 6)	MW U value (comparison of medians)
Demographics	Age	55 (44 - 66)	52 (29 - 74)	17
	Sex (F/M)	4/2	2/4	
	Ethnicity	6x white European	6x white European	
	Smoker / ex smoker <sup>1</sup>	0/0	0/0	
Disease	Months since diagnosis	0 (0 - 0)	17 (9 - 95)	
	Concomitant drugs	nil	nil	
	Maximum disease extent			
	Left sided total	0 0	2 4	
Clinical Scores	Baron score	0 (0 - 0)	3 (3-3)	
	Mayo Score	1 (1 - 3)	9 (5 - 12)	
	Truelove and Witts	4x normal	2x severe 2x moderate 2x mild	
Clinical investigation	Deviation of Hct (%)	1.9 (1.6 - 6.6)	8.1 (6.3 - 10.5)	4
	Weight loss (%)	0.0 (0.0 - 0.0)	0.8 (0.4 - 3.6)	0
	Temperature >37.5°C	nil	nil	
	Pulse >90 bpm	nil	nil	
	Hb	145 (129 - 145)	121 (104 - 147)	7.5
	ESR	9.0 (8.0-10.0)	36.0 (33.0-39.0)	0
	CRP	2.0 (1.0 - 3.5)	15.5 (4.5-31.8)	2
	Raised Creatinine <sup>2</sup> Albumin	nil 45 (41 - 46)	nil 37 (35 - 42)	

Table 26. Patient characteristics for microarray experiments in UC. Values given as medians (IQR), significance between groups calculated using the Mann Whitney U test. <sup>1</sup>has not smoked within 3 months of biopsy, <sup>2</sup>above laboratory reference range.

The UC microarray experiments included 6 patients in both the active sigmoid UC group and the control group. Patients are matched for age ( $p = 0.898$ ) and ethnicity, although the proportion of females is higher in the control group compared with active UC, 66% versus 33% respectively.

Four of the active UC group had total UC; all had Baron Grade 3 macroscopic inflammation at the site of biopsy. Markers of clinical activity that are significantly different between active UC and the control group include; deviation of Hct ( $p = 0.024$ ), weight loss ( $p = 0.002$ ), ESR ( $p = 0.029$ ), CRP ( $p = 0.038$ ). Markers of clinical activity that are not significantly different between active UC and controls include; Hb ( $p = 0.762$ ) and albumin ( $p = 0.143$ ).

	Control (n = 6)	Active Crohn's disease (n = 6)	MW U value (comparison of medians)	
Demographics	Age*	55 (44 - 66)	52 (39 - 60)	14
	Sex (F/M)	4/2	3/3	
	Ethnicity	4x white European	4x white European	
	Smoker / ex smoker <sup>1</sup>	0/0	0/0	
	Reason for scope	3x rectal bleed 2x change of bowel habit 1x abnormal CT	2x surveillance 1x change of bowel habit 1x abdominal pain 1x flare of symptoms	
Disease	Months since diagnosis	0 (0 - 0)	90 (15 - 220)	
	Concomitant drugs	nil	nil	
	Disease phenotype			
	Ileocolonic	0	4	
	Colonic	0	2	
	Unknown	0	0	
Previous operations	0	0		
Clinical Scores	Harvey Bradshaw Index	1 (0 - 4)	7 (0 - 20)	8
	CDAI	53 (35 - 60)	158 (60 - 255)	7
	Biopsy site	6x normal	5x ulcers 1x bleeding but no ulcers	
Clinical investigation	Deviation of Hct (%)	1.9 (1.6 - 6.6)	4.9 (2.5 - 6.5)	7
	Weight loss (%)	0.0 (0.0 - 0.0)	3.8 (0.0 - 13.7)	12
	Temperature >37.5°C	nil	nil	
	Pulse >90 bpm	nil	nil	
	Hb	145 (129 - 149)	133 (130 - 136)	4
	ESR	6 (3 - 8)	22 (13 - 28)	3
	CRP	2 (1 - 3)	40 (7 - 54)	3
	Raised Creatinine <sup>2</sup>	nil	nil	
Albumin	45 (41 - 46)	35 (35 - 38)	1	

Table 27. Patient characteristics for microarray experiments in Crohn's Disease. Values given as medians (IQR), <sup>1</sup>has not smoked within 3 months of biopsy, <sup>2</sup>above laboratory reference range.

In the Crohn's microarrays the groups are matched for age ( $p = 0.619$ ) and ethnicity although there are slightly more females in the control group compared

## Chapter 4. The miRNA signature of IBD

with active Crohn's, 66% versus 50% respectively. None of the patients had undergone previous resections.

Although clinical activity scores tended to be higher in patients with active Crohn's, there is no statistical significance between active Crohn's disease and healthy control as measured by the Harvey Bradshaw Index ( $p = 0.128$ ), or the Crohn's disease Activity Index ( $p = 0.093$ ). Interestingly although the mucosa is ulcerated at the site of biopsy in 5 out of the 6 patients with Crohn's and haemorrhagic in the other, only 17% (1/6) have HBI >16 and thus categorised as severe, and only 50% (3/6) have a CDAI >150.

Biochemical markers of disease severity include Hb and Alb which have similar values in the active Crohn's and control groups. Although the other markers of disease severity tend towards showing an inflammatory reaction there is no statistical difference between active Crohn's and the healthy control groups, deviation of Hct ( $p = 0.091$ ), weight loss ( $p = 0.454$ ), Hb ( $p = 0.629$ ), ESR ( $p = 0.062$ ), CRP ( $p = 0.056$ ) and albumin ( $p = 0.114$ ).

### 4.3.1 The expression of miR-31, miR-146b and miR-223 are consistently increased in active sigmoid Ulcerative Colitis.

For each active UC array card the relative expression of individual miRNAs are normalised to MammU6 and compared against the expression in the healthy control microarrays. The miRNAs are ranked in order of relative expression for each array card pair and assigned a rank. Table 28 shows those miRNAs which have the greatest increase in relative expression in UC compared with healthy controls.

There are 3 miRNAs which are consistently ranked in the top 20 miRNAs for each array card pair; miR-31, miR-146b and miR-223. There are 11 others which are ranked in the top 20 for 2 of the 3 array card pairs, but these are not considered further.

The mean ( $\pm$  s.d., one tailed unpaired students t test) fold change in the expression of miR-31, miR-146b and miR-223 compared with healthy control is 35.257 ( $\pm$  0.782

s.d.,  $p < 0.001$ ),  $3.766 (\pm 1.223 \text{ s.d.}, p = 0.076)$  and  $8.523 (\pm 2.424 \text{ s.d.}, p = 0.047)$  respectively.

Of the 377 miRNA analysed using the TaqMan™ microarray card A (v2.0), 150 miRNAs amplified with a  $CT \leq 35$  in all of the 3 array card pairs, appendix 8.3.1. Of those miRNAs which amplified in the microarrays, 13.3% (20/150) had a mean increased expression compared with healthy controls of  $\geq 2.0$  fold. This is 5.3% (20/377) of the total number of miRNAs contained in the microarray card, and consistent with previously published results (Wu et al., 2008).

Array card pair 1	Array card pair 2	Array card pair 3	
miRNA	miRNA	miRNA	rank
miR-31	miR-31	miR-31	1
miR-486	miR-132	miR-223	2
miR-223	miR-15b	miR-636	3
miR-483	miR-223	miR-708	4
miR-146b	let-7d	miR-135b	5
miR-449a	let-7b	miR-628	6
miR-202	miR-135b	miR-193a	7
miR-155	miR-125b	miR-142-5p	8
miR-708	miR-181a	miR-331	9
miR-146a	miR-146b	miR-132	10
miR-193b	miR-212	miR-18b	11
miR-150	miR-138	miR-494	12
miR-454	miR-20a	miR-146b	13
miR-539	miR-155	miR-483	14
miR-874	miR-21	miR-142-3p	15
miR-29b	miR-99a	miR-21	16
miR-126	miR-142-3p	miR-99a	17
miR-212	miR-193b	miR-548d	18
miR-628	miR-152	let-7f	19
miR-140	miR-126	miR-885	20

Table 28. Microarray for active UC, top ranked miRNA. The miRNAs are ranked in order of expression. miRNA expressions are normalised to mammU6. Each array card is compared against a healthy control array card. This table shows the 20 most increased miRNAs in each of the array card pairs. **Dark grey** = those miRNAs which feature in all three of the array card pairs. **Light gray** = those miRNAs which feature in two of the three array card pairs.

### 4.3.2 The expression of miR-194, miR-200b, miR-375 and miR-422a are consistently decreased in active sigmoid Ulcerative Colitis.

There are 4 miRNAs which are consistently ranked in the 20 miRNAs that have the most decreased relative expression for each array card pair; miR-194, miR-200b, miR-375 and miR-422a. There are 8 other that are ranked within the 20 miRNAs with the most decreased relative expression for 2 of the 3 array card pairs, but these are not considered further.

Array card pair 1	Array card pair 2	Array card pair 3	rank
miR-145	miR-375	miR-422a	1
miR-492	miR-422a	miR-141	2
miR-328	let-7a	miR-375	3
miR-339	miR-200b	miR-429	4
miR-27b	miR-489	miR-192	5
miR-127	miR-192	miR-194	6
miR-451	miR-194	miR-339	7
miR-375	miR-134	miR-200b	8
miR-99a	miR-196b	miR-200b	9
miR-30b	miR-200a	miR-200a	10
miR-422a	miR-141	miR-370	11
miR-149	miR-106b	miR-491	12
miR-133b	miR-9	miR-10b	13
miR-429	miR-200b	miR-130b	14
miR-218	miR-339-3p	miR-197	15
miR-423	miR-339-5p	miR-411	16
miR-143	miR-191	miR-532	17
miR-200b	miR-370	miR-376c	18
miR-194	miR-425	miR-486	19
miR-103	miR-486	miR-186	20

Table 29. Microarray for active UC, bottom ranked miRNA. The miRNAs are ranked in order of expression. miRNA expressions are normalised to mammu6. Each UC array card is compared against a healthy control array card. This table shows the 20 most decreased miRNAs in each of the array card pairs. **Dark grey** = those miRNAs which feature in all three of the array experiments. **Light grey** = those miRNAs which feature in two of the three array experiments.

The mean ( $\pm$  s.d., one tailed unpaired students t test) fold change in the expression of miR-194, miR-200b, miR-375 and miR-422a compared with healthy control is

0.213 ( $\pm$  0.058 s.d.,  $p = 0.020$ ), 0.251 ( $\pm$  0.124 s.d.,  $p = 0.014$ ), 0.129 ( $\pm$  0.045 s.d.,  $p = 0.002$ ) and 0.106 ( $\pm$  0.005 s.d.,  $p < 0.001$ ) respectively.

There are 33 miRNA whose expression is more than halved in the sigmoid mucosa of active UC compared with healthy controls, this is 22.0% (33/150) of miRNAs which amplified or 8.8% (33/377) of the total number of miRNAs contained on the microarray card, appendix 8.3.1.

### 4.3.3 Candidate miRNA expression in Crohn’s Disease by microarray.

Array card pair 1	Array card pair 2	Array card pair 3	
miRNA	miRNA	miRNA	rank
miR-31	miR-31	miR-494	1
miR-451	miR-202	miR-22	2
miR-486-5p	miR-142-5p	miR-32	3
miR-486-3p	miR-494	miR-636	4
miR-223	miR-636	miR-142-5p	5
miR-548d-5p	let-7c	miR-579	6
miR-15a	miR-618	miR-130b	7
miR-20b	miR-22	miR-15a	8
miR-18b	miR-486-3p	miR-652	9
miR-193a-3p	miR-579	miR-487a	10
miR-331-5p	miR-29b	miR-202	11
miR-135b	miR-18b	miR-455-5p	12
miR-16	miR-487a	miR-29b	13
miR-636	miR-224	miR-34a	14
miR-32	miR-142-3p	miR-885-5p	15
miR-21	miR-625	miR-223	16
miR-518b	miR-135b	miR-452	17
miR-708	miR-148a	miR-618	18
miR-142-5p	miR-135a	miR-518b	19
miR-625	miR-182	miR-370	20

Table 30. Microarray in Crohn’s disease, top ranked miRNA. The expressions of the miRNAs are normalised to MammU6. Each active sigmoid CD array is compared against a healthy control. **Dark grey** = those miRNAs which feature in the top 20 for all three of the array card pairs. **Light grey** = those miRNAs which feature in two of the three array card pairs. **Yellow** = those miRNAs which are also identified as candidate miRNAs for UC.

Only miR-142-5p is consistently increased in the top 20 miRNAs for active sigmoid Crohn's. Of the three candidate miRNAs that are increased in UC, only miR-31 and

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miR-223 appear in 2 out of the 3 array card pairs. In the 3<sup>rd</sup> array card pair they feature at ranks 121<sup>st</sup> and 25<sup>th</sup> respectively, Table 30. The mean relative expressions of miR-31 and miR-223 across the array card pairs are 39.288 fold ( $\pm$  26.914 s.d.,  $p = 0.109$ ) and 6.001 fold ( $\pm$  1.504 s.d.,  $p = 0.020$ ) respectively.

Only miR-1 is identified by microarray to have an expression which is consistently decreased across all 3 array card pairs. Of the 4 candidate miRNAs identified to have a decreased expression in UC by microarray, only miR-422a is ranked in the 20 miRNAs with the lowest relative expression in Crohn's. The miR-422a is ranked 18<sup>th</sup>, 15<sup>th</sup> and 35<sup>th</sup> with a mean relative expression of 0.700 ( $\pm$  0.224 s.d.,  $p = 0.120$ ).

Array card pair 1	Array card pair 2	Array card pair 3	rank
miRNA	miRNA	miRNA	
miR-361-5p	miR-204	miR-95	1
miR-125a-3p	miR-125a-3p	miR-1	2
miR-487b	miR-489	miR-361-5p	3
miR-193a-5p	miR-491-5p	miR-671-3p	4
miR-324-5p	miR-134	miR-323-3p	5
miR-190	miR-125a-5p	miR-454	6
miR-338-3p	miR-149	miR-134	7
miR-335	miR-574-3p	miR-23b	8
miR-147b	miR-193b	miR-16	9
miR-133b	miR-331-3p	let-7a	10
miR-376c	miR-331-5p	miR-125a-5p	11
miR-10b	miR-376a	miR-628-5p	12
miR-218	miR-133b	miR-342-3p	13
miR-1	miR-16	miR-489	14
miR-885-5p	miR-422a	miR-374b	15
miR-202	miR-1	miR-365	16
miR-133a	miR-186	miR-20a	17
miR-422a	miR-24	miR-204	18
miR-23b	miR-133a	miR-181c	19
miR-192	miR-138	miR-301b	20

Table 31. Microarray in Crohn's disease, bottom ranked. The 20 miRNAs with the most decreased expression in Crohn's disease. This table shows the 20 most decreased miRNAs from each of the 3 array card pairs. **Dark grey** = those miRNAs which feature in all three of the array experiments. **Light grey** = those miRNAs which feature in two of the three array experiments. **Yellow** = those miRNAs which are identified as candidate miRNAs in the UC microarray.

In those candidate miRNAs whose relative expressions are decreased in active UC compared with healthy controls, the mean relative expressions for miR-194, miR-

200b and miR-375 across the Crohn's disease array card pairs are 1.588 fold ( $\pm 0.746$  s.d.,  $p = 0.234$ ), 1.577 fold ( $\pm 0.806$  s.d.,  $p = 0.246$ ) and 0.851 fold ( $\pm 0.183$  s.d.,  $p = 0.220$ ) respectively.

### 4.4 The expression of candidate miRNAs in the sigmoid mucosa of treatment naïve active and inactive Ulcerative Colitis.

In the previous section, microarrays identified seven candidate miRNAs which are differentially expressed in active sigmoid UC compared with healthy sigmoid mucosa. Reverse Transcription-qPCR was conducted to validate the expression of miR-31, miR-146b, miR-194, miR-200b, miR-223, miR-375 and miR-422a in active and inactive sigmoid UC compared with healthy controls.

		Control (n = 21)	Inactive UC (n = 19)	(U value, p value)	Active UC (n = 25)	(U value, p value)
Demographics	Age	59 (45 - 75)	55 (49 - 66)	118, p = 0.452	49 (36 - 62)	165.5, p = 0.118
	Sex (F/M)	11/10	7/8		8/13	
	Ethnicity (if not white)		1x Asian		2x Asian	
	Smoker / Ex Smoker <sup>1</sup>	0/1	0/1		0/6	
Disease	Months since diagnosis	0 (0 - 0)	156 (125 - 221)		60 (6 - 42)	
	Concomitant drugs disease extent (maximal/today) <sup>2</sup>	21 x nil	19 x nil		25 x nil	
	normal	21/21	0/19		0/0	
	Left sided total	0/0	10/0		17/14	
Clinical Scores	Baron score	0 (0 - 0)	0 (0 - 0)		3 (3 - 3)	
	Mayo Score	1.5 (0 - 9)	1 (0 - 2)		6 (4 - 11)	
	Truelove and Witts	16x normal 4x mild 1x moderate	14x normal 5x mild		12x mild 8x moderate 5x severe	
Clinical investigation	Deviation of Hct (%)	4.0 (3.1 - 9.4)	3.2 (1.4 - 4.9)	44.5, p = 0.255	7.2 (3.9 - 8.3)	110.5, p = 0.422
	Weight loss (%)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	124.5, p > 0.999	0.0 (0.0 - 2.1)	128, p = 0.089
	Temperature >37.5°C	nil	nil		nil	
	Pulse >90 bpm	nil	nil		nil	
	Hb	139 (128 - 149)	145 (142 - 154)	36, p = 0.092	130 (114 - 147)	115.5, p = 0.534
	ESR	6 (2 - 9)	10 (4 - 16)	40.5, p = 0.580	42 (19 - 45)	16.5, p = 0.029
	CRP	2.0 (1.0 - 3.5)	5.0 (3.3 - 6.8)	14.5, p = 0.062	15 (6 - 34)	31, p = 0.002
Raised Creatinine <sup>3</sup>	nil	nil		nil		
Albumin	41 (38 - 44)	44 (42 - 44)	42, p = 0.363	38 (37 - 42)	84, p = 0.112	

Table 32. Patient characteristics for the candidate miRNA RT-qPCR. Values given as medians (IQR). Statistical relevance of the difference between the median values and the control group are calculated using the two tailed Mann Whitney U test, probability statistics are shown as the (U value, p value). <sup>1</sup>have not smoked within 3 months of biopsy. <sup>2</sup>maximal distribution of disease known before endoscopy / disease distribution today. <sup>3</sup>above laboratory reference range.

The characteristics of the control, active UC and inactive UC groups are given in Table 32. The median age of the healthy control is 59 years (IQR 45 – 75), the median age of the active UC and inactive UC groups are 49 years (IQR 36 – 62) and 55 years (IQR 49 – 66) respectively. There is no significant difference in the ages of the active and inactive UC groups compared with healthy controls. The median time since diagnosis for the active UC group is 60 months (IQR 6 – 42) and the inactive UC group, 156 months (IQR 125 – 221).

The following traditional measures of disease activity are not significantly different in active UC compared with the healthy controls; haematocrit ( $p = 0.422$ ), weight loss ( $p = 0.089$ ), haemoglobin ( $p = 0.534$ ) and albumin ( $p = 0.112$ ). These same measures are also not statistically different between active and inactive UC. Traditional measures of activity in which there is a significant difference between active UC and healthy controls are the ESR ( $p = 0.029$ ) and CRP ( $p = 0.002$ ). Between active and inactive UC there is a statistically significant difference in the ESR ( $p = 0.042$ ), but not the CRP ( $p = 0.054$ ).

#### 4.4.1 The expressions of miR-31 and miR-223 are increased in the sigmoid colon in both active and inactive UC.

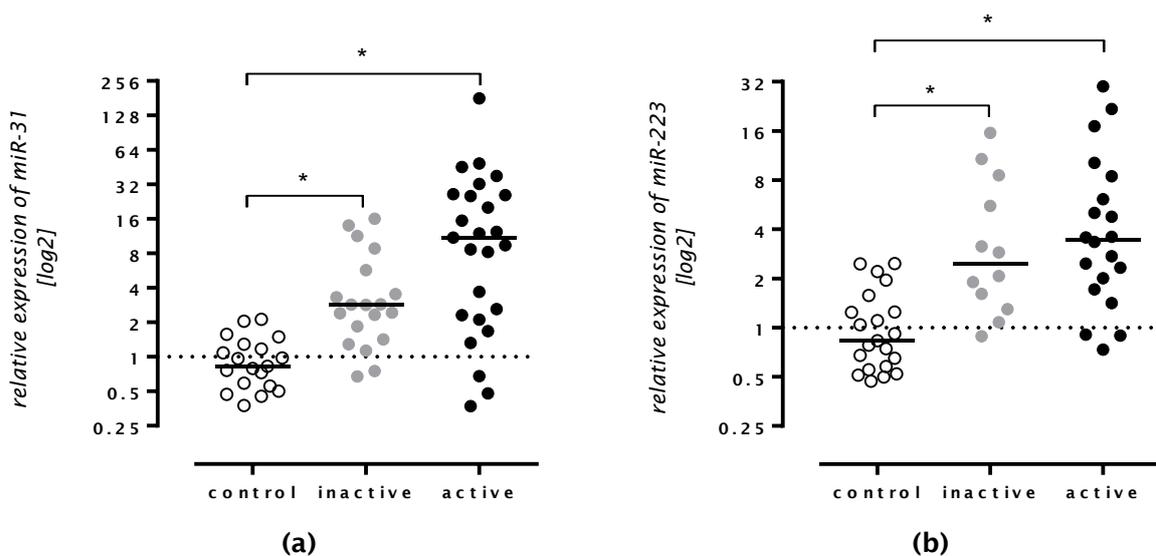


Figure 31. The relative expressions of miR-31 and miR-223 in sigmoid UC by RT-qPCR. The relative expression of (a) miR-31 and (b) miR-223 is increased in both active ( $n = 25$ ) and inactive ( $n = 19$ ) sigmoid UC. Expression of miRNAs was analysed by RT-qPCR. miRNA expression

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was normalised to RNU44 and compared to healthy control (n = 21). Median values were compared using the two tailed Mann Whitney U test. \* P < 0.05.

The relative expressions of miR-31 and miR-223 are increased in the sigmoid mucosa in both active and inactive UC compared with healthy controls. The expression of miR-31 is increased by a median of 2.830 (IQR 1.419 – 5.726) fold in inactive UC (p < 0.001) and 10.970 (IQR 2.214 – 26.110) fold in active UC (p < 0.001). The expression of miR-223 is significantly increased by a median 2.477 (IQR 1.377 – 7.801) fold in inactive UC (p < 0.001), and 3.454 (IQR 1.790 – 7.853) fold in active UC (p < 0.001).

There is also a statistically significant difference between the expression of miR-31 in active versus inactive UC (p = 0.029), but not in the expression of miR-223 between active and inactive UC (p = 0.546).

### 4.4.2 The expression of miR-146b is increased in the sigmoid colon of active but not inactive UC.

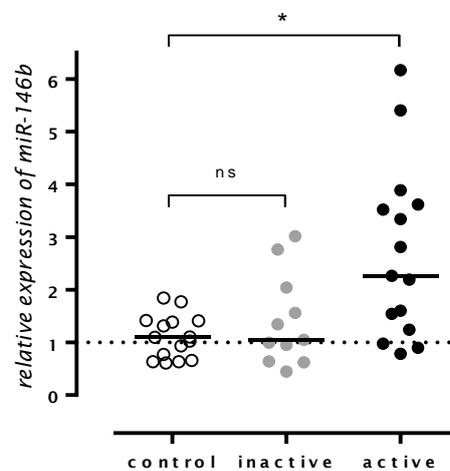


Figure 32. The relative expression of miR-146b in sigmoid UC by RT-qPCR. The relative expression of mir-146b is increased in active but not inactive sigmoid UC. Expression of miRNA was analysed by RT-qPCR. miRNA expression was normalised to RNU44 and compared to healthy control. Median values were compared using the two tailed Mann Whitney U test. \* P < 0.05. ns = not significant.

The relative expression of miR-146b is significantly increased by 2.261 (IQR 1.243 - 3.619) fold in the mucosa of active sigmoid UC ( $p = 0.002$ ). The expression of miR-146b is not significantly altered in inactive UC compared with healthy control, fold increase 1.048 (IQR 0.642 - 2.044,  $p = 0.678$ ).

There is a significant difference in the expression of miR-146b in active UC compared with inactive UC,  $p = 0.029$ .

#### 4.4.3 The expressions of miR-194, miR-200b, miR-375 and miR-422a are reduced in the sigmoid colon of patients with active UC.

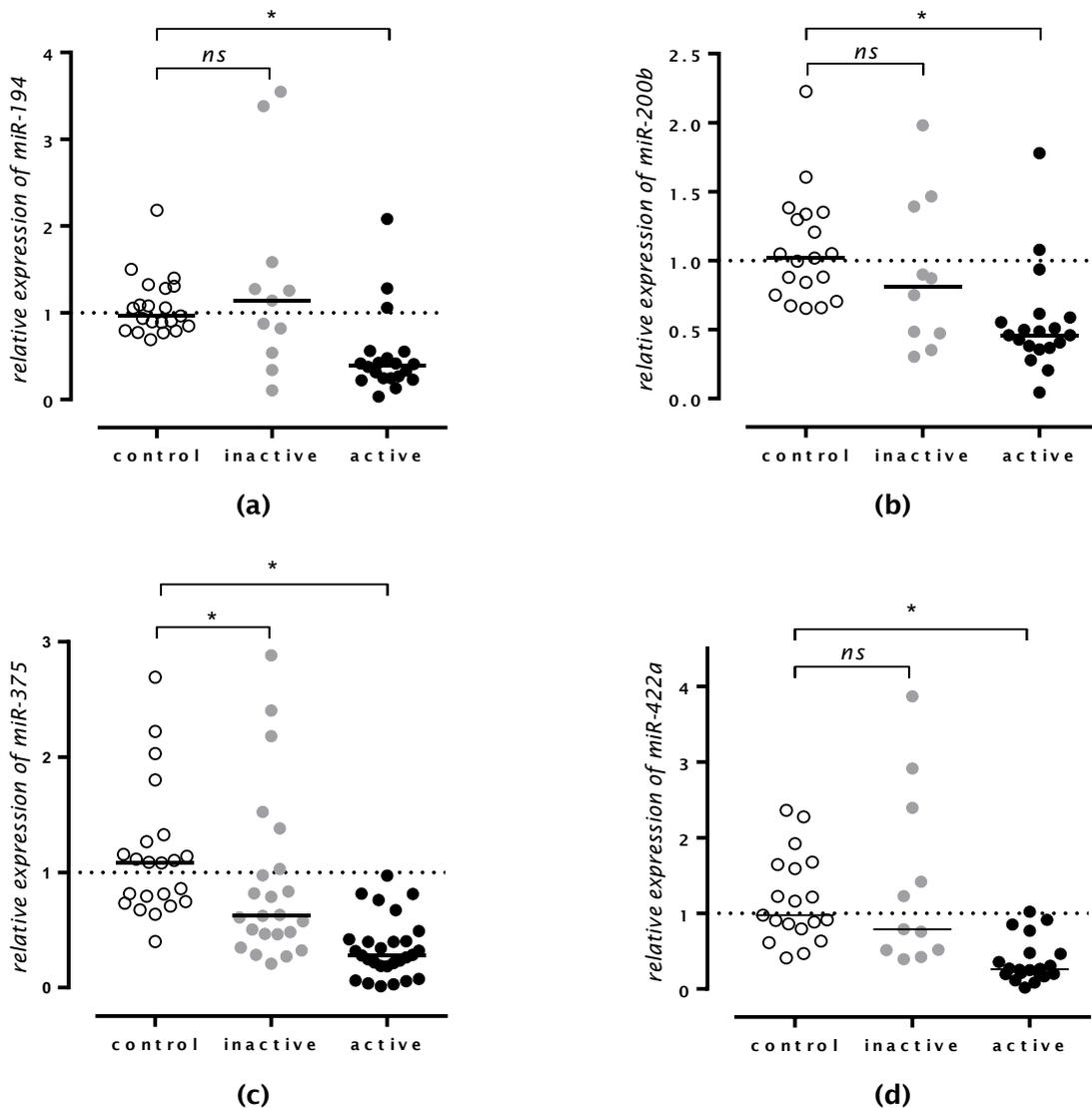


Figure 33. The relative expression of miR-194, miR-200b, miR-375 and miR-422a is decreased in sigmoid UC. The relative expressions of (a) miR-194, (b) miR-200b, (c) miR-375 and (d) miR-422a are

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decreased in active sigmoid UC. The relative expression of miR-375 is also decreased in inactive sigmoid UC. Expression of miRNAs is normalised to RNU44 and compared with healthy control. Median values are compared using the two tailed Mann Whitney U test. \* P < 0.05, ns = not significant.

The relative expressions of miR-194, miR-200b, miR-375 and miR-422a are significantly decreased in active sigmoid UC compared with healthy controls.

The relative expression of miR-194 is decreased by a median of 0.392 (IQR 0.247 – 0.534,  $p < 0.001$ ) fold in the mucosa of active sigmoid UC compared with healthy controls. However there is no statistically significant difference in the expression of miR-194 in inactive sigmoid UC compared with healthy controls, fold difference 1.140 (IQR 0.539 – 1.584,  $p = 0.937$ ). There is also a significant difference in the relative expression of miR-194 in active UC compared with inactive UC,  $p = 0.011$ .

The relative expression of miR-200b is decreased by a median of 0.460 (IQR 0.367 – 0.588) fold in the mucosa of active sigmoid UC compared with healthy controls ( $p < 0.001$ ). However there is no significant difference in the relative expression of miR-200b in the mucosa of inactive sigmoid UC compared with healthy controls, fold difference 0.812 (IQR 0.443 – 1.411,  $p = 0.281$ ) or in the expression of miR-200b between active and inactive sigmoid UC,  $p = 0.124$ .

The relative expression of miR-375 is decreased in the mucosa of both active and inactive sigmoid UC. The expression of miR-375 is significantly decreased by 0.281 (IQR 0.187 – 0.415,  $p < 0.001$ ) fold in the mucosa of active sigmoid UC, and by 0.624 (IQR 0.462 – 1.030,  $p = 0.021$ ) fold in the mucosa of inactive sigmoid UC compared with healthy control. There is a significant difference between the expressions of miR-375 in active and inactive sigmoid UC,  $p = 0.001$ , indicating that although reduced in inactive UC compared with healthy control there is a greater decrease in active UC.

The relative expression of miR-422a is decreased by a median of 0.261 (IQR 0.196 – 0.472,  $p < 0.001$ ) fold in the mucosa of active sigmoid UC compared with healthy control. However there is no significant difference in the expression of miR-422a in the mucosa of inactive sigmoid UC compared with healthy controls, fold

difference 0.789 (IQR 0.513 - 2.393,  $p = 0.731$ ). There is a significant difference between the expressions of miR-422a in active and inactive sigmoid UC,  $p = 0.001$ .

## **4.5 The differential expressions of certain miRNAs are dependent on the current treatment regime.**

The interpretation of published studies into the profile and / or the expressions of miRNAs in IBD are made difficult by confounding factors and group heterogeneity, prominent amongst these is the current treatment. Current therapy for IBD targets the immune response and since miRNAs have been shown to influence, and be influenced by many aspects of the immune response, it stands to reason that existing treatments for IBD could influence miRNA expression, function or both. Thus current treatment differences challenge the ability to interpret existing studies on the subject.

The expressions of miR-31 and miR-375 have been shown to be increased and decreased respectively in the mucosa of patients with active sigmoid UC and Crohn's. Using miR-31 and miR-375 as representative of the most differentially expressed miRNA in IBD the aim is to compare the miRNA expressions of miR-31 and miR-375 in the sigmoid mucosa in relation to the current treatment regime.

### **4.5.1 The expression of miR-31 is significantly different in patients taking Azathioprine compared with 5-ASA alone**

In patients with Baron grade 3 macroscopically inflamed active sigmoid UC the expression of miR-31 in patients taking Azathioprine is significantly lower than patients taking 5-ASA alone ( $p = 0.005$ ) and patients taking no treatment ( $p = 0.001$ ). There is no difference in the expression of miR-31 between patients on no treatment and those taking 5-ASA ( $p = 0.198$ ), Figure 34 "active".

In patients who have inactive UC there is a significantly lower expression of miR-31 in patients on Azathioprine compared with those on 5-ASA alone ( $p = 0.016$ ) although the reduced expression of miR-31 in patients on Azathioprine is not significantly less than patients on no treatment ( $p = 0.071$ ), Figure 34 "inactive".

In both active and inactive UC the expression of miR-31 is not significantly different in patients taking Infliximab compared with patients taking Azathioprine, 5-ASA alone or no treatment.

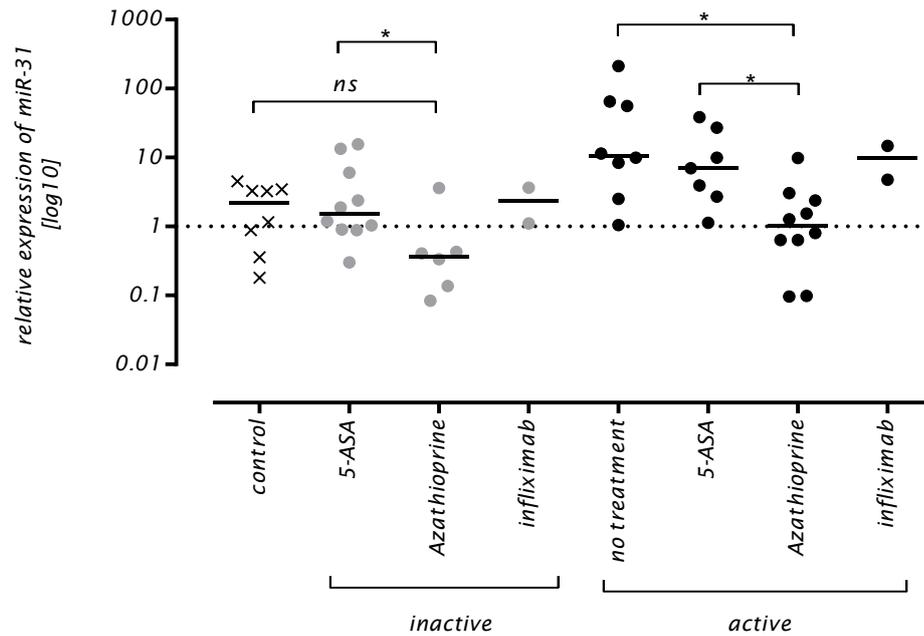


Figure 34. The effect of treatment of miR-31 in UC. The mucosal expression of miR-31 is decreased in patients taking Azathioprine in both inactive and active UC compared with patients on other treatment regimes. The expressions of miR-31 in sigmoid biopsies taken from patients with active and inactive UC and taking a variety of treatments are compared by RT-qPCR. The expression of miR-31 is normalised to RNU44 and presented as relative expression compared against patients with inactive UC and taking no treatment (control). Median values were compared with the one tailed Mann-Whitney U test. \*  $p < 0.05$ , ns = not significant. U values for each comparison are found in appendix 8.6.2.

In patients with actively inflamed sigmoid Crohn's the expression of miR-31 is significantly lower in patients taking Azathioprine ( $p = 0.025$ ) and significantly increased in patients taking Infliximab alone ( $p = 0.014$ ) compared with patients taking no treatment, Figure 35 “active”.

In patients with a previous history of sigmoid Crohn's but no active colonic inflammation, the expression of miR-31 in the sigmoid is higher in patients taking Azathioprine compared with patients taking no treatment but this is not significant ( $p = 0.062$ ), Figure 35 “inactive”.

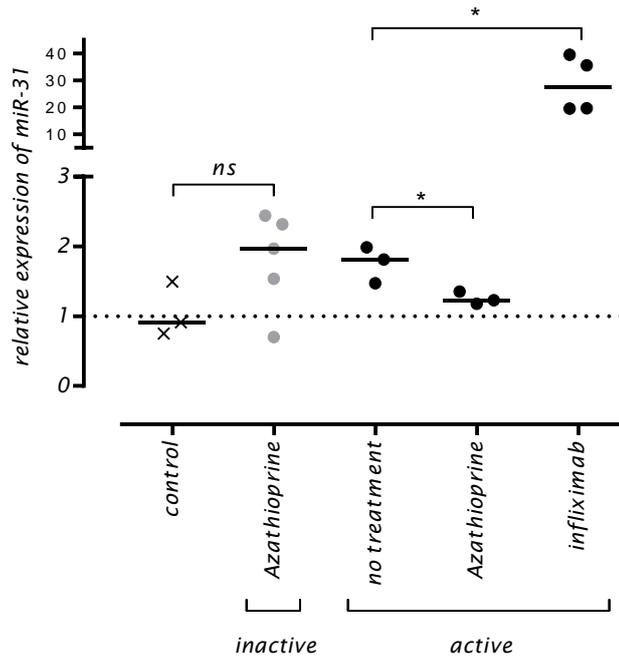


Figure 35. The effect of treatment on miR-31 in Crohn's disease. The expression of miR-31 is less in patients taking Azathioprine with active Crohn's compared with patients with the same degree of inflammatory activity on different treatment regimes. The expressions of miR-31 in sigmoid biopsies taken from patients with active and inactive Crohn's disease and on a variety of treatments are compared by RT-qPCR. The expression of miR-31 is normalised to RNU44 and presented as relative expression compared against patients with inactive Crohn's disease and taking no treatment (control). Median values were compared with the two tailed Mann-Whitney U test. \*  $p < 0.05$ , ns = not significant.

#### 4.5.2 The expression of miR-375 is not significantly different in patients taking Azathioprine compared with 5-ASA alone

Although the relative expression of miR-375 is decreased in the sigmoid mucosa of patients with inactive and active UC compared to healthy controls, there is no significant difference in the expression of miR-375 within either of the inactive or active sigmoid UC groups, when divided into treatment groups, Figure 36.

Neither 5-ASAs, Azathioprine or Infliximab appear to alter the expression of miR-375.

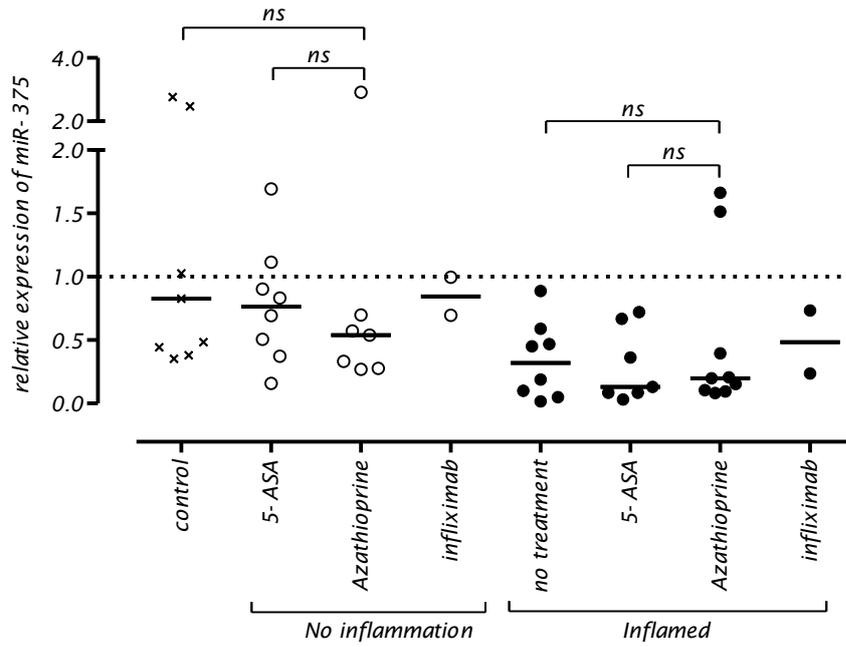


Figure 36. The expression of miR-375 is not altered in patients on different treatment regimes for UC. The expressions of miR-375 in sigmoid biopsies taken from patients with active and inactive UC and on a variety of treatments are compared by RT-qPCR. The expression of miR-375 is normalised to RNU44 and presented as relative expression compared against patients with inactive UC and taking no treatment (control). Median values are compared with the two tailed Mann-Whitney U test. ns = not significant.

## 4.6 Bioinformatic search for microRNA targets

Bioinformatic searches were conducted using the 2 databases; TargetScan version 5.2, June 2011 (Lewis et al., 2003, Garcia et al., 2011) and MirTarget2 v4.0 (Wang and El Naqa, 2008, Wang, 2008) both accessed through to 28<sup>th</sup> Sept 2011.

TargetScan uses the MiRanda algorithm which primarily predicts miRNA targets through the base pair matching qualities between the seed region of the miRNA and the 3' UTR of the target gene.

MirTarget2 (available at [www.mirdb.org](http://www.mirdb.org)) uses a support vector machine to predict targets based on known gene / miRNA interactions.

	TargetScan (conserved targets)	MirTarget2 (score >50)	Total number of targets	% similarity (targets predicted by both)
miR-31	233	247	411	21
miR-146b	129	229	323	12
miR-194	252	333	496	19
miR-200b	798	844	1302	26
miR-223	202	240	373	23
miR-375	141	35	170	4
miR-422a	13	199	210	1

Table 33. Comparison of the predicted target results for 2 distinct bioinformatic search algorithms. (TargetScan v5.2, and MiTarget v4.0).

The number of predicated targets using the 2 algorithms is shown in Table 33. By combining 2 distinctly separate methods of identifying gene targets it is hoped that there is a decrease in false positive results, and increase the true positive results.

Bioinformatic searches are limited in their ability to predict useful targets and therefore the results presented are those predicted targets that are ranked in the top 20 for TargetScan v5.2, those with a MirTarget2 score >80 and those of potential interest after review of all the targets and there known functions.

The aims are to identify potential gene targets for miR-31, miR-146b, miR-194, miR-200b, miR-223, miR-375 and miR-422a. As well as identify a potential target that is targeted by more than one of the candidate miRNAs.

#### 4.6.1 Targets of interest for miR-31

Target gene	Target scan					MirTarget2		pathway
	rank	conserved sites total	poorly conserved sites total	Total context score	Aggregate P <sub>CT</sub>	rank	target score	
SH2D1A	3	2	0	-0.74	0.49	9	88	Encodes a protein that inhibits SLAM and modifies T cell signal transduction
PRKCE	6	1	2	-0.66	0.55	28	77	Key role in TLR4 / LPS mediated signaling in activated macrophages and possibly INF regulation (Aksoy et al., 2004).
STARD13	15	1	0	-0.52	0.52	148	59	Suppresses cell growth in liver cancer, inhibits activity of Raf-1-ERK1/2-p70S6K. Selectively activates CDC42 and is involved in TGF- $\beta$ pathway
CD28	107	1	1	-0.27	0.41			required for T cell activation
PPP3CA	160	1	0	-0.2	0.21			activates T cells
NCK2	191	1	0	-0.16	0.21			involved in the regulation of receptor protein kinases
TSLP	192	0	1	-0.10	<0.1			T <sub>H</sub> 2 pathways, crosstalk between DCs and macrophages
IL1R1						87	67	inflammation
CDC42EP4						108	64	CDC42 regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression.
SP1						132	61	Involved in TGF- $\beta$ pathway

Table 34. Targets of interest for miR-31 Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009).

Mir-31 potential targets numerous genes involved with aspects of the immune response, Table 34. Genes of interest include; PRKCE and SH2D1A.

PRKCE, (protein kinase C $\epsilon$ ) is essential for IL-12 synthesis and therefore involved in the T<sub>H</sub>1 response. PRKCE plays a role in LPS stimulation of macrophages and DCs through the TLR4 receptor pathway (Aksoy et al., 2004, Aksoy et al., 2002)

SH2D1A encodes a protein that inhibits SLAM (signaling lymphocyte-activation molecule) by blocking the recruitment of SHP-2 to its docking site; this and other surface molecule interactions can modify cell signal transduction in activated T, B and NK cells. Mutations in this gene cause lymphoproliferative syndrome X-linked

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type 1 or Duncan disease, a rare immunodeficiency characterized by extreme susceptibility to infection, suggesting that inhibition of SH2D1A can exaggerate T cell signalling and thus inflammation.

### 4.6.2 Targets of interest for miR-146b

target gene	Target scan					MirTarget2		pathway
	rank	conserved sites total	Poorly conserved sites total	Total context score	Aggregate P <sub>CT</sub>	rank	target score	
TRAF6	2	1	1	-0.99		2	98	This protein functions as a signal transducer in the NF- $\kappa$ B pathway by mediating signals of the TNF receptor superfamily, members of the Toll/IL-1 family and receptors such as CD40, TNFSF11/RANCE and IL-1. This protein also interacts with various protein kinases including IRAK1/IRAK, SRC and PKC $\zeta$ .
CD80	6	1	0	-0.44		109	61	Macrophages in mice show increased expression, blockade of CD80 reduces colitis
IRAK1	23	1	0	-0.77		12	87	This gene encodes the IL-1 receptor-associated kinase 1, one of two serine/threonine kinases that become associated with IL1R upon stimulation. This gene is partially responsible for IL1-induced up-regulation of the transcription factor NF- $\kappa$ B.
CCBP2	27	1	0	-0.56				Chemokines and their receptor-mediated signal transduction are critical for the recruitment of effector immune cells to the inflammation site.
SMAD4	40	1	0	-0.4		18	82	SMAD proteins are activated by transmembrane serine-threonine receptor kinases in response to TGF- $\beta$ . The product of this gene forms complexes with other activated Smad proteins, which accumulate in the nucleus and regulate the transcription of target genes. Mutations or deletions in this gene have been shown to result in pancreatic cancer, juvenile polyposis syndrome, and hereditary hemorrhagic telangiectasia syndrome
ATG12	46	1	0	-0.38				ATG16 GWAS study
CARD10	54	1	1	-0.35				GWAS study

Table 35. Targets of interest for miR-146b Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009).

MiR-146b has been shown to have an influence on the TLR / NF- $\kappa$ B pathways; TRAF (particularly TRAF 6) protein family has been extensively studied in mice and peripheral blood lymphocytes. CD80 is involved in the co-stimulatory signal essential for T-lymphocyte activation. T-cell proliferation and cytokine production is induced by the binding of CD28 or CTLA-4 to this receptor. IRAK1 binds to the IL-1 receptor, triggering intracellular signalling cascades leading to up-regulation and mRNA stabilization.

## 4.6.3 Targets of interest for miR-194

Target gene	Target scan					MirTarget2		pathway
	rank	Total conserved sites	Total poorly conserved sites	Total context score	Aggregate P <sub>cr</sub>	rank	mirdb.org target score	
ITPKB	7	1	1	-0.65	0.52	56	76	Regulates immune cell function and is required for T and B cell development.
ACVR2B	18	2	0	-0.52	0.75	73	74	ACVR2B is an activin type 2 receptor and belongs to the (TGF-β) superfamily.
MAPK1	56	1	1	-0.41	0.5			Acts as a transcriptional repressor of INF-γ induced genes. Seems to bind to the promoter of CCL5, DMP1, FIH1, IFITM1, IRF7, IRF9, LAMP3, OAS1, OAS2, OAS3 and STAT1. MAPKs regulate a wide variety of cellular functions including; signals from growth factors, differentiation, proliferation and phosphorylation of many inflammatory transcription factors.
FOXO1	221	1	0	-0.13	0.41			A member of the forkhead family. The specific function of this gene has not yet been determined; however, it may play a role in myogenic growth and differentiation.
TSLP		0	1	-0.03	<0.1			T <sub>H</sub> 2 pathways

Table 36. Targets of interest for miR-194 Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009).

## 4.6.4 Targets of interest for miR-200b

Target gene	TargetScan				MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	rank	mirdb.org target score	
ZEB2	1	6	0	-2.13	1	100	Interacts with receptor-mediated, activated SMADs. Represses transcription of E-cadherin
RECK	10	2	1	-0.95	5	99	The expression is suppressed in many. In normal cells, this membrane glycoprotein may serve as a negative regulator for MMP-9, a enzyme involved in tumour invasion and metastasis
ZEB1	29	2	0	-0.79	3	100	Represses T cell specific IL2 gene. Represses E-cadherin, and induces an epithelial-mesenchymal transition (EMT) by recruiting SMARCA4/BRG1. Promotes tumorigenicity by repressing miRNAs
SULF1	62	1	1	-0.65	56	84	Acts as a negative regulator of TGF-β, widely involved with inflammation. facilitates apoptosis
PLCG1	311	1	0	-0.36			T cell receptor signalling
MAP3K1	323	1	0	-0.35			sapk / jnk, activated p38 MAPK, TLR pathways
TOB1	502	1	0	-0.26			TGF-β signalling
XIAP	570	1	0	-0.23			Apoptosis
SMAD2	603	1	0	-0.21			TGF-β signalling
FYN	614	1	0	-0.2			B cell receptor signalling
PTEN	622	1	0	-0.2			Inhibitor of apoptosis

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Target gene	TargetScan				MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	rank	mirdb.org target score	
ACVR2B	627	1	1	-0.19			TGF- $\beta$ signalling
EP300	666	1	0	-0.17			TGF- $\beta$ signalling
CREBBP	675	1	0	-0.16			TGF- $\beta$ signalling
ACVR2A	725	1	0	-0.13			TGF- $\beta$ signalling
PRKAB1	753	1	0	-0.12			Autophagy
SP1	758	1	0	-0.11			TGF- $\beta$ signalling
BCL2	782	1	0	-0.05			Inhibitor of apoptosis, autophagy and death receptor signalling
MEF2D	796	1	0	0.03			Activating p38 MAPK pathways

Table 37. Targets of interest for miR-200b Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009).

### 4.6.5 Targets of interest for miR-223

Target gene	TargetScan					MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	Aggregate P <sub>CT</sub>	rank	target score	
FBXW7	1	1	1	-1.36	0.86	1	99	Inactivation results in repression of TGF- $\beta$ dependent transcription
RHOB	4	1	1	-0.73	0.51	13	87	Role in heat stress response and increased transcriptional activity of NF- $\kappa$ B
FOXO1	7	1	2	-0.66	0.55	41	75	A member of the forkhead family. The specific function of this gene has not yet been determined; however, it may play a role in myogenic growth and differentiation.
ACVR2A	41	1	0	-0.43	0.47	11	88	Activins are growth and differentiation factors which belong to the TGF- $\beta$ superfamily.
CALML4	47	1	0	-0.41	0.47	115	62	Ca binding protein involved in inflammation
AK2	93	1	2	-0.33	0.62			Gene identified by GWAS. Function in T <sub>H</sub> 17 cells
SP1	105	1	0	-0.29	0.46			regulates the expression of a large number of genes involved in a variety of processes such as cell growth, apoptosis, differentiation and immune responses
MMP16	170	1	1	-0.16	0.58			Also targeted by miR-146b in glioma cells. Expressed by T cells.
TSLP						5	89	T <sub>H</sub> 2 pathways

Table 38. Targets of interest for miR-223 Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009).

### 4.6.6 Targets of interest for miR-375

Target gene	TargetScan				MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	rank	target score	
ATG2B	61	1	0	-0.16			associated with gastric and colorectal cancers through autophagy. regulates the expression of a large number of genes involved in a variety of processes such as cell growth, apoptosis, differentiation and immune responses, TGF-β signalling.
SP1	126	1	0	-0.06			

Table 39. Targets of interest for miR-375 Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009).

Although TSLP is not identified as a target of miR-375 by either TargetScan or MirTarget2, miR-375 has been shown to play a role in IL-13 induced expression of TSLP in HT29 cells (Biton et al., 2011).

### 4.6.7 Targets of interest for miR-422a

Target gene	TargetScan				MirTarget2		pathway
	rank	total conserved targets	total poorly conserved targets	Total context score	rank	target score	
SULF1	3	1	0	-0.41	36	71	Acts as a negative regulator of TGF-β, widely involved with inflammation. facilitates apoptosis Increased expression improves survival in sepsis, by abrogating sepsis induced lymphocyte apoptosis.
DYRK1A	8	1	1	-0.27			
TSLP		0	1	-0.06			T <sub>H</sub> 2 pathways This protein functions as a signal transducer in the NF-κβ pathway by mediating signals of the TNF receptor superfamily, members of the Toll/IL-1 family and receptors such as CD40, TNFSF11/RANCE and IL-1. This protein also interacts with various protein kinases including IRAK1/IRAK, SRC and PKCζ.
TRAF6					67	65	

Table 40. Targets of interest for miR-422a Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009).

#### 4.6.8 TSLP as a target

There are 3285 potential targets identified for the 7 candidate miRNAs, using TargetScan and MirTarget2, at least 45 of which have been shown to play a role within inflammatory pathways involved in IBD and are shown in Table 34 to Table 40. A number of genes are potential targets for >1 candidate miRNA. TSLP and SP1 are targets for 4 miRNAs, and TRAF6, ACVR2B, FOXO1, SULF1 and ACVR2A are all targets for 2 miRNAs.

Using TargetScan v6.0 to predict gene targets for the candidate miRNAs shows that TSLP is a target for miR-31, miR-194 and miR-422a. Base pair matching between the seed region of the miRNAs and TSLP, and context scores using the TargetScan algorithm is shown in Table 41. Predicting which miRNA is most likely to target TSLP using the same algorithm reveals that miR-495 has the highest context score. However miR-495 did not amplify in all microarray experiments for UC and therefore does not feature in the miRNA expression tables, Table 28 and Table 29. The expression of miR-495 in active Crohn's is 1.547 fold greater than healthy control, although is ranked 64<sup>th</sup> in order of relative expression compared with healthy control, appendix 8.3.3.

TSLP is an interesting target for miRNAs in UC due to its association with T<sub>H</sub>2 inflammation, and its involvement in epithelial homeostasis. TSLP was therefore chosen ahead of SP1, a gene which encodes the transcription factor Sp1. Through the regulation of a vast number of genes Sp1 has many functions including differentiation, proliferation and apoptosis. The main role for Sp1 is in embryological development, although through its effect on cell growth and proliferation deregulation of Sp1 is associated with certain forms of cancer. It is not known to have an association with inflammation (Deniaud et al., 2009).



## 4.7 Chapter discussion

Experimental models have shown a crucial role for miRNA in many inflammatory pathways and suggest a fundamental role for miRNA in the aetiology of IBD. However, the currently available miRNA profiles for IBD provide an ambiguous picture that is difficult to interpret. Interpretation and clinical usefulness of a miRNA profile largely depends on the sample phenotype as this relates to treatment decisions and a range of long term outcomes. The wide variety of phenotypes is also likely to reflect subtle differences in aetiological factors which may influence miRNA expression.

The lack of consistent evidence requires that the first step is to determine which miRNAs are differentially expressed in active IBD in a homogeneous and clinically applicable patient group. The focus here is on UC, with a comparison to the miRNA signature in Crohn's.

One of the important clinical and sample selection criteria revolve around current therapy. Medical treatments for IBD target elements of the immune system, traditional treatments such as steroids and thiopurines act non-specifically on immune function through multiple pathways. New treatments such as Infliximab act against specific targets, in this case TNF- $\alpha$ , however the effects of altered TNF- $\alpha$  function will influence a multitude of other downstream pathways. It therefore stands to reason that therapy alters or interacts at some level with miRNA expression and/or function. Therefore patients are only included in this study if they have not had medical therapy for IBD for  $\geq 6$  months, termed "treatment naive". In section 4.5 it is shown that patients taking Azathioprine have a decreased level of miR-31 compared with macroscopically matched patients taking no medication or 5-ASA alone. This effect is not seen with miR-375 and may represent a miRNA specific phenomenon; however it does indicate that patients on heterogeneous treatment regimes should not be grouped together for the purposes of measuring miRNA levels. In both the study by Wu et al. (2008) and Fasseu et al. (2010) patient groups were heterogeneous and included patients on disease modifying agents in 80% and 63% of cases respectively.

There are two other important features of sample homogeneity; the first is the origin of the tissue. Biopsies are collected from endoscopy and not surgical resections due to the obvious differences in disease phenotype, activity and the likely differences in aetiological or propagating factors. The second is the region from which the samples were taken. There is debate over regional differences in miRNA expression, in particular, miR-422b was one of six miRNAs to have a difference in expression between different regions of the colon (Wu et al., 2010). Again this may reflect either a functional role for certain miRNAs and / or an interaction with aspects of the colonic environment, for example the microbiome or homeostasis mechanisms, which also have regional differences. For consistency biopsies are taken only from the sigmoid colon.

Inflammatory activity is measured clinically in 3 ways, symptomatically, biochemically and endoscopically. In traditional clinical practice the logistics of endoscopy result in clinical activity being measured predominantly by a combination of symptom activity and biochemical results. However symptoms are subjective and correlate poorly with endoscopic activity due to a wide variety of confounding factors, including psychosocial issues and coexisting dietary intolerances. Very raised biochemical marker levels are well validated at distinguishing severe disease from mild to moderate disease and are independent predictors of a poor outcome or the need for colectomy. However despite being more objective than symptoms, biochemical markers still correlate poorly with endoscopic activity (Turner et al., 2011) or changes in activity over time. Endoscopic activity is therefore becoming the predominant factor in grading disease activity and disease response to treatment both clinically and in clinical trials.

In this study patients were included in the active UC group if they had Baron grade 3 inflammation at the time of biopsy. Patients with active UC tended towards a higher clinical activity score although as Table 32 shows, there are a number of interesting points; in both the control group and the inactive UC group there are patients who report symptoms which score them as having mild and moderate disease activity despite no macroscopic inflammation, and in the active UC group

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only 20% (5/25) had a “severe” Truelove and Witts score, despite severe macroscopic inflammation.

There are a number of clinical activity scoring systems for UC (D'Haens et al., 2007), all are subjective and many open to inter-observer variation. The Truelove and Witts score was designed to capture those patients with acute severe UC, it is the oldest, arguably the simplest and the most commonly used clinical score, thus it is favoured in this study to compare clinical activity between patient groups. However, the lack of concordance between endoscopic activity and the Truelove and Witts score is explained by some of its weaknesses; its focus on haematochezia as a required symptom renders it unable to capture those patients who do not view their stools or are not passing frequent amounts of bloody stools, and the inclusion of signs and symptoms that are also frequently seen in non-IBD pathologies such as haematochezia caused by haemorrhoids or diarrhoea predominant Irritable Bowel Syndrome, reduces its specificity. Alternative scoring systems are available, but their use is limited in clinical practice.

Biochemical markers compared in this study include; haematocrit, haemoglobin, albumin and the inflammatory markers ESR and CRP. Only the CRP and ESR were significantly different between active UC and healthy control. The ESR in active UC is 42 compared with 6 in healthy controls,  $p = 0.029$ . The CRP is also increased, 15 versus 2 respectively,  $p = 0.002$ . Despite the statistical significance between CRP in active disease and healthy control, approximately 25% (6/25) of the active UC group had a CRP within the normal range, a sensitivity for active inflammation in UC which is consistent with published results (Henriksen et al., 2008). Endoscopic activity is the only reliable and objective way of measuring disease activity and should be the standard way of measuring disease activity in UC.

The strength of this study is the homogeneous selection criteria, based on a definition of Baron grade 3 active sigmoid UC in patients who are naive to disease modifying treatment.

The number of miRNAs is doubling every 3 years, when last updated there were 1600 human miRNA hairpin precursors and 2042 mature miRNA sequences

registered on the miRBase sequences database (release 19, available at [www.mirbase.org](http://www.mirbase.org), assessed 30<sup>th</sup> November 2012).

Rapidly increasing numbers of miRNA make complete miRNA profiling impossible. When first performed the TaqMan® Array card v2.0 analysed approximately 40% (377) of known miRNAs (miRBase, release 14), at time of writing this is only 20% (377/1921). However, the 377 miRNAs included on the array card are considered the “*most highly characterised*” and “*of demonstrated interest to the research community*” by the manufacturer (Applied Biosystems, 2010), implying that they are the most likely miRNAs to play a prominent role in IBD.

During microarray experiments approximately 50% of miRNAs do not amplify, (Wu et al., 2010, Fasseu et al., 2010). During these experiments only 40% (150/377) miRNAs amplified within the cut off Ct value < 35. There are many factors that cause no or poor amplification including loss of small RNA, < 200 nucleotides, during the precipitation step of the TRIzol® extraction, or poor amplification due to the complexity of the array card, acknowledged by the manufacturer. Although alternative RNA extraction techniques and pre-amplification steps are available, the relative expression is only compared between the same miRNAs in active IBD and control and not between different miRNAs in the same samples; therefore the quantification of the quality and efficacy of small RNA extraction is not necessary.

There are two prominent methods that are used to present the expression of miRNAs in array studies; the first is by listing in order of relative expression between the active group and a control (Wu et al., 2008, Wu et al., 2010, Fasseu et al., 2010) using the  $2^{-\Delta\Delta Ct}$  calculation (Livak and Schmittgen, 2001), the second calculates the relative expression but presents the results in rank order, from the miRNA with the greatest increase in relative expression to that with the greatest decrease in relative expression. Presenting the relative expression values can be misleading as they often differ from the actual relative expression by many fold.

Validation of microarrays is always necessary because of the inherent variation in biological samples and the inaccuracy of microarray results. It is a feature of microarrays that when validated with RT-qPCR up to 50% of the differentially expressed miRNAs are no longer significant. Microarray data therefore provides

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only “candidate miRNAs”. To reduce the error rate firstly a method of “pairwise analysis” was performed similar to that used by Van der Goten et al. (2012), a technique which involves the comparison of one active IBD array card versus one control array card, this was repeated 3 separate times. For each pair of cards the differential expression using the  $2^{-\Delta\Delta Ct}$  was calculated and expressions ranked.

The 20 miRNAs with the greatest increase or decrease in relative expression from each pair were then compared to discover which miRNA are consistently the most differentially expressed in UC. The same experiments were performed for Crohn's.

Focusing on UC, for logistical reasons, seven miRNAs have been identified as consistently differentiated in the sigmoid mucosa of active UC versus healthy controls. At the time of performing the microarray there are 65 miRNAs that have been shown to have a differential expression in the colonic mucosa of patients with UC by array (Wu et al., 2008, Fasseu et al., 2010, Van der Goten et al., 2012), see Table 13. At the time of the experiment only miR-31, miR-223, miR-375 and miR-422a had been previously shown to be increased in the mucosa of UC. However in a recently published abstract, Van der Goten et al. (2012) have also shown the differential expression of miR-146b, miR-194 and miR-200b in UC.

MiR-31 is well established to have increased expression in many forms of cancer including; lung (Liu et al., 2010b), stomach (Zhang et al., 2010b), colon (Creighton et al., 2010) and interestingly UC associated dysplasia but not the background UC (Olaru et al., 2011). It has been identified as an up-regulated miRNA in UC in some studies (Fasseu et al., 2010, Van der Goten et al., 2012), but not others (Wu et al., 2008).

Most of the functional pathways influenced by miR-31 have so far implicated it in oncogenesis, these include LATS2 and PPP2R2A (Liu et al., 2010b), which suppress pathways involved with lung cancer, and SATB2 in tumour associated fibroblasts which reduces the ability of metastases to migrate and invade (Aprelikova et al., 2010), HIF transcription factors (Liu et al., 2010a), and many others.

MiR-31 plays an important role in the immune regulation and responses. TNF- $\alpha$  increases miR-31 expression in epithelial cells which then target SELE and ICAM-1

decreasing neutrophil binding (Suarez et al., 2010). MiR-31 was also found to directly target the FOXP3 transcription factor and is under-expressed in T<sub>regs</sub> compared with other T cell phenotypes (Rouas et al., 2009). The role of miR-31 in IBD is yet to be established.

MiR-146 has a well established role in a number of inflammatory pathways in which its influence appears to be part of a negative feedback regulator. The expression of miR-146 is induced through TLR activation and the NF- $\kappa$ B pathway, whilst its increased expression targets TRAF6 and IRAK1, negatively influencing the TLR / NF- $\kappa$ B pathway (Bhaumik et al., 2008). A negative feedback role is also suggested by an increased expression of miR-146 by TNF- $\alpha$  and IL-1 $\beta$  on one hand (Nakasa et al., 2008) whilst down regulating TNF- $\alpha$  and IL-1 $\beta$  on the other (Jones et al., 2009).

The pathological relevance of miR-146 has been demonstrated in various cancers including breast cancer in which miR-146 has been shown to suppress NF- $\kappa$ B and influence the migration of breast cancer cells (Bhaumik et al., 2008). In inflammatory conditions miR-146 has been shown to have an increased expression including Rheumatoid Arthritis (Nakasa et al., 2008), Osteoarthritis (Jones et al., 2009) and alveolitis. In the later, rapid induction of IL-1 $\beta$  induces miR-146 which negatively regulates the release of IL-8 and CCL5, factors crucial to the innate immune response, thus again dampening the immune response.

The expression of miR-146 is induced in THP-1 cells (Taganov et al., 2006) and human cord blood monocytes (Lederhuber et al., 2011) by LPS stimulation suggesting a role in innate immune response and a potential role within the response to luminal flora. The influence of miR-146 on intestinal immunology has not yet been investigated.

The expression of miR-194 is increased in the mouse lung after stimulation with LPS and is associated with reduced levels of TNF- $\alpha$ , in the lung miR-194 is not affected by corticosteroids (Moschos et al., 2007). In a caco-2 intestinal epithelial cell model, hepatocyte nuclear factor-1 $\alpha$  (HNF-1 $\alpha$ ) induces expression of miR-194, therefore suggesting an influence on epithelial cell differentiation (Hino et al., 2008). The function of miR-194 has yet to be established.

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MiR-200 is expressed by epithelial cells and is implicated in many cancers due to its influence on epithelial-mesenchymal transition which is required for metastases (Paterson et al., 2008). Differences in miR-200 expression have been associated with enhanced tumorigenesis, significantly correlated with decreased survival and are associated with resistance to several chemotherapy drugs; docetaxel in non-small cell cancer cells, cisplatin in breast cancer cells, and gemcitabine in cholangiocarcinoma cells. The role in inflammation and /or IBD has yet to be established.

The influence of miR-223 can be broken down into three broad overlapping areas; its de-regulated expression in solid organ cancers, its role in haematological malignancies and its effect on lymphocyte function.

MiR-223 is deregulated in colorectal cancer (Earle et al., 2010), bladder cancer (Gottardo et al., 2007), ovarian cancer (Laios et al., 2008), increased in oesophageal adenocarcinoma (Mathe et al., 2009), oesophageal squamous cell cancer (Zhang et al., 2010a), small cell lung cancer (Miko et al., 2009) and down regulated in hepatocellular carcinoma.

The prominent role for miR-223 is in granulopoiesis. Overall miR-223 negatively regulates progenitor proliferation and granulocyte differentiation and activation through interaction with numerous transcription factors which are either targets of miR-223 or target miR-223 directly including *mef2c* (Johnnidis et al., 2008b), *stathmin 1* (Wong et al., 2008), *LMO2* (Malumbres et al., 2009), *AML1/ETO*, (Fazi et al., 2007) and *E2F1* (Pulikkan et al., 2010). Abnormalities of miR-223 expression are manifested pathologically as lymphoproliferative disorders (Bellon et al., 2009), polycythemia vera mononuclear cells (Bruchova et al., 2008), myelofibrosis, essential thrombocythaemia (Hussein et al., 2009) and most prominently, T cell acute lymphoblastic leukaemia (Chiaretti et al., 2010) (Mi et al., 2007).

As well as tumorigenesis and granulopoiesis, miR-223 also has an important influence on inflammation across many different tissues. MiR-223 is increased in the synovial fluid (Murata et al., 2010) and deregulated in peripheral T lymphocytes (Fulci et al., 2010) in Rheumatoid Arthritis. MiR-223 is deregulated in muscle inflammation and around sites of spinal cord injury (Nakanishi et al.,

2010), in hepatic inflammation (Yu et al., 2009) and reduced in the Systemic Inflammatory Response Syndrome, SIRS (Wang et al., 2010).

Overall, miR-223 appears to function as a regulator of the inflammatory process, reduced levels of miR-233 are associated with hypersensitive neutrophils (Johnnidis et al., 2008a), and although decreased levels prevents macrophage hyper-activation it primes macrophages for a stronger pro-inflammatory response (Li et al., 2010).

Importantly miR-223 is differentially expressed between monocytes and monocyte derived DCs (Cekaite et al., 2010) and may account for some of the characteristics of the mature cell types.

MiR-375 has been shown to be decreased in UC miRNA array by Wu et al. (2008). It has been implicated in breast, hepatic and gastric cancer. In gastric cancer miR-375 is down-regulated and directly targets the apoptosis pathway through PDK1 (Tsukamoto et al., 2010) or JAK2 (Ding et al., 2010). Interleukin 13 induces TSLP expression via a miR-375 dependent pathway in HT29 cells indicating that miR-375 plays a role in the T<sub>H</sub>2 response and epithelial crosstalk (Biton et al., 2011).

MiR-422 has been shown to be decreased in UC miRNA array by Wu et al. (2008), although has no known functional role in inflammation. Limited literature reveals a possible role in bile acid synthesis (Song et al., 2010) and bone regeneration (Palmieri et al., 2008).

One of the complexities of predicting miRNA targets by bioinformatic searches is the quantity of data that is produced. Using TargetScan v5.2 and MirTarget2 v4.0 together generated 3285 potential targets for the 7 miRNAs. Genes which are ranked in the top 20 for TargetScan v5.2, or have a score above 80 for MirTarget2 v4.0 or have a significant known inflammatory function are highlighted in Table 34 to Table 40.

Bioinformatic searches using TargetScan identified Thymic Stromal Lymphopoietin (TSLP) as a potential target for miR-31, miR-194 and miR-422a, Table 41. Using

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MirTarget2, TSLP is also a predicted target for miR-223, with target score of 89. There is little concordance between TargetScan and MirTarget2.

The seed site base pairing pattern has been discussed in section 2.7.3.1. A 7mer-1A seed match indicates that there is an exact Watson Crick base pairing of positions 2-7, followed by an Adenine, of the mature miRNA 5' end and the 3'UTR of TSLP. The 4 miRNAs bind to the positions on the 3' end of TSLP at the positions shown in Table 41.

The focus on TSLP as a target for 4 of the miRNAs is also fuelled by recent interest in TSLP as a mediator of the  $T_H2$  response seen in respiratory diseases. Abnormal lymphocyte function is thought to be central to the pathogenesis of IBD with UC favouring a  $T_H2$  inflammatory response. Thus TSLP is an interesting target gene to investigate further.

It has so far been demonstrated that there is a differential expression of 7 miRNAs in mucosal biopsies taken from patients with active UC; to date the published miRNA profiles in UC have used whole tissue samples via either biopsies or resection specimens (Wu et al., 2008, Fasseu et al., 2010). A limitation of analysing miRNA expression and / or function when miRNA profiling using whole tissue samples is that they consist of a number of cell types which are likely to express different levels of miRNA depending on their function and local environment (Lagos-Quintana et al., 2002). A method of identifying which cell types within the mucosa express individual miRNAs, so called "spatial expression patterns" is in situ hybridisation, a technique which allows localisation of specific segments of nucleic acids within a histological specimen. Using in situ hybridisation, in their seminal paper, Wu and colleagues (2008) localised the altered expression of miR-192 and MIP-2 $\alpha$  in active UC to colonic epithelial cells.

Despite the potential advantages of identifying which cells within the biopsies express the candidate miRNAs, in situ hybridisation has its challenges. Previous experience within the laboratory had been unsuccessful in identifying miRNAs by in situ hybridisation so the alternative approach of analysing miRNA expression directly in cell lines which represent 3 prominent cell types within the mucosa and

which feature as predominant factors in the aetiology of IBD; the epithelial cell (HT29), monocyte (THP-1) and lymphocyte (Jurkat), was used.

Not localising miRNA expression within tissue samples is a weakness of this study, previous laboratory experience of localising miRNA expression in tissue by in situ hybridisation had been unsuccessful, however newer refined techniques are proving more sensitive and specific (Thompson et al., 2007, Deo et al., 2006). In hindsight developing a successful technique and adding this step to the study protocol may have led to alternative avenues of investigation and may have strengthened the link between miRNA expression and TSLP in mucosal lymphocytes, a subject discussed in subsequent chapters.



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**Chapter 5. The expression of candidate  
miRNA in mucosal lymphocytes**

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## 5.1 Chapter abstract

Abnormal lymphocyte function is thought to be central to the pathogenesis of IBD with UC favouring a  $T_H2$  inflammatory response. Previous studies have identified 65 miRNAs that have a deregulated expression in the mucosa of UC, however, there is a paucity of research into miRNA expressions within individual mucosal cell subtypes including mucosal lymphocytes.

Sigmoid mucosal lymphocytes are extracted from whole mucosal biopsies by Percoll centrifugation. The pattern of candidate miRNA expressions when analysed with RT-qPCR is similar to that seen in whole mucosal biopsies. Although only the differential expression of miR-31, -194, -200b and -223 reach significance with fold differences of 2.07, 0.33, 0.41 and 11.41 respectively.

Pro-inflammatory  $CD4^+CD25^{\text{intermediate}}$  lymphocytes were then separated from the lymphocyte pool using surface antigen staining characteristics and flow cytometry. In the  $CD4^+CD25^{\text{intermediate}}$  lymphocyte subset the expressions of miR-31, miR-146b and miR-223 are increased significantly at 3.69, 155.8 and 6.34 fold respectively. The altered expressions of the other candidate miRNAs; miR-194, miR-200b, miR-375 and miR-422a did not reach statistical significance.

The results presented in this chapter show the differential expression of certain miRNA in mucosal lymphocytes and the  $CD4^+CD25^{\text{intermediate}}$  pro-inflammatory lymphocyte subset. A number of the candidate miRNAs are not significantly altered within these mucosal cell subsets, a finding that agrees with the assertion that the expressions of miRNAs are cell type specific.

## 5.2 Chapter introduction

To date 72 miRNAs have been shown to be either increased or decreased in the mucosa of active IBD compared with healthy mucosa, of which 65 are deregulated in UC (Wu et al., 2008, Wu et al., 2010, Fasseu et al., 2010, Van der Goten et al., 2012). These miRNA signatures however are taken from the colonic mucosa of a heterogeneous group of patients and the studies are poorly concordant as discussed in previous chapters. In earlier chapters it is shown that in treatment naive actively inflamed sigmoid UC there are 7 consistently deregulated miRNAs.

There is growing evidence that miRNA expression is not only organ specific but also cell specific (Rouas et al., 2009, Hezova et al., 2009), for reasons that may include a conserved functionality in specific cell types. With the growing evidence that miRNAs play a crucial role in cell functionality and inflammatory pathways defining the miRNA expression in specific cell types is crucial to understanding the pathogenesis of UC

The GI tract mucosa consists of numerous cell types, the imbalance, abnormal interaction and function of which have all been associated with UC. Predominant cells of the epithelium are the epithelial cell, dendritic cell and lymphocyte. In this chapter the expression of the candidate miRNAs is shown, firstly in an enriched suspension of mucosal lymphocytes and then in the CD4<sup>+</sup>CD25<sup>intermediate</sup> pro-inflammatory subset.

The lymphocyte has been chosen for a number of reasons; following the validation of 7 candidate miRNA in mucosal biopsies, it has been shown by in-silico analysis that TSLP is a target of 4 of the candidate miRNAs. Interestingly TSLP is associated with T<sub>H</sub>2 disorders of the lung and skin and has been previously associated with UC (Tanaka et al., 2010). TSLP is thought to be mainly an epithelial cell derived factor which influences dendritic cells and the function of down-stream lymphocytes, conferring a non-inflammatory environment and thus supporting epithelial homeostasis.

Recent data reviewed by Takai (2012) would suggest that TSLP is more widely expressed than previously thought, the expression of TSLP has not been

previously described in lymphocytes, although it has been shown that peripheral naive CD4<sup>+</sup> lymphocytes upon activation express the TSLP receptor (Rochman et al., 2007). Since abnormal lymphocyte function and loss of the regulatory mechanisms that support epithelial homeostasis is a hallmark of UC, the association between lymphocytes, miRNA expression and TSLP in UC is interesting and unknown.

Initially mucosal lymphocytes are extracted from the sigmoid mucosa using density centrifugation and later CD4<sup>+</sup>CD25<sup>intermediate</sup> mucosal lymphocytes are extracted from sigmoid biopsies by density centrifugation followed by sorting from other cell subtypes using surface antigen staining characteristics and flow cytometry. The expressions of the candidate miRNAs are analysed with RT-qPCR.

### 5.3 The expression of candidate miRNA in mucosal lymphocytes

There are clear differences in the number and function of mucosal lymphocytes in IBD compared with healthy individuals. The association of abnormal miRNA expression and lymphocyte function has been clearly demonstrated in experimental models and peripheral lymphocyte subsets, although not in human mucosal biopsies.

The abnormal expression of a distinct group of miRNA in whole mucosal biopsies has been demonstrated. The aim of this section is to demonstrate the expression of those previously described candidate miRNAs in mucosal lymphocytes.

		Control (n = 4)	Active UC (n = 4)	MW U value (comparison of medians)
Demographics	Age	76 (61 - 89)	53 (46 - 62)	1.5, p = 0.086
	Sex (F/M)	3 / 1	3 / 1	-
	Ethnicity (if not white)	nil	nil	-
	Smoker / ex smoker <sup>1</sup>	0 / 0	0 / 0	-
Disease	Months since diagnosis	0 (0 - 0)	151 (0 - 334)	-
	Concomitant drugs	4 x nil	4 x nil	-
	disease extent (maximal/today) <sup>2</sup>			-
	normal	4/4	0/0	-
	Left sided total	0/0	3/0	-
Clinical Scores	Baron score	0 (0 - 0)	3 (3 - 3)	-
	Mayo Score	2 (1 - 4)	11 (7 - 15)	-
	Truelove and Witts	3x normal 1x mild	2x mild 1x moderate 1x severe	-
	Deviation of Hct (%)	7.0 (4.5 - 11.8)	4.0 (2.4 - 7.3)	3, p = 0.400
Clinical investigation	Weight loss (%)	0.0 (0.0 - 5.0)	0.0 (0.0 - 0.0)	6, p > 0.999
	Temperature >37.5°C	nil	nil	-
	Pulse >90 bpm	nil	nil	-
	Hb	126 (99 - 153)	125 (117 - 131)	6, p > 0.999
	ESR	4 (3 - 5)	29 (12 - 60)	0, p = 0.029
	CRP	5 (1 - 14)	52 (27 - 223)	0, p = 0.029
	Raised Creatinine <sup>3</sup>	1	nil	-
Albumin	41 (27 - 44)	33 (24 - 40)	4, p = 0.371	

Table 42. Patient characteristics for the mucosal lymphocyte extraction. Values given as medians (IQR), <sup>1</sup>has not smoked within 3 months of the biopsy. <sup>2</sup>maximal distribution of disease known before endoscopy / disease distribution today. <sup>3</sup>above laboratory reference range. Comparisons of the medians was calculated with the two tailed Mann Whitney U test, probability statistics are shown as the U value and p value.

Lymphocytes were extracted from the sigmoid mucosa using EDTA, collagenase and a discontinuous Percoll® gradient as outlined in section 2.3.1. The relative expressions of miRNAs were quantified by RT-qPCR relative to healthy controls.

In section 5.4, it is shown that Percoll centrifugation results in a suspension in which 32.2% of cells stain CD3<sup>+</sup>.

Cell suspensions from healthy mucosa (n = 4) are compared against active UC (n = 4). Patients were matched for age and sex. All active UC patients had Baron Grade 3 inflammation at the site of the biopsy, for the full demographical information see Table 42. Traditional measures of disease activity that are not statistically different between the groups are deviation of haematocrit (p = 0.400), percentage weight loss (p > 0.999), haemoglobin (p > 0.999) and albumin (p = 0.371).

The inflammatory markers ESR and CRP have significantly higher values in active UC compared with healthy controls, p = 0.029 and p = 0.029 respectively.

The relative expressions of the 7 candidate miRNAs in the cell suspension gained from Percoll centrifugation follows the same trend as found in whole mucosal biopsies. The 3 miRNAs that have an increased expression in whole mucosal biopsies also have an increased expression in the cell suspension, and the 4 miRNAs that have a decreased expression in whole mucosal biopsies also have a decreased expression in the cell suspension.

The expression of miR-31 is 2.070 (IQR 1.690 - 2.162) fold and the expression of miR-223 is 11.41 (IQR 11.37 - 12.88) fold in active UC compared with healthy controls. Both of these increases are statistically significant, p = 0.029 and p = 0.029 respectively.

Although the expression of miR-146b is increased by 1.269 (IQR 1.137 - 1.665) fold, this does not reach significance, p = 0.114.

## Chapter 5. MicroRNA in mucosal lymphocytes

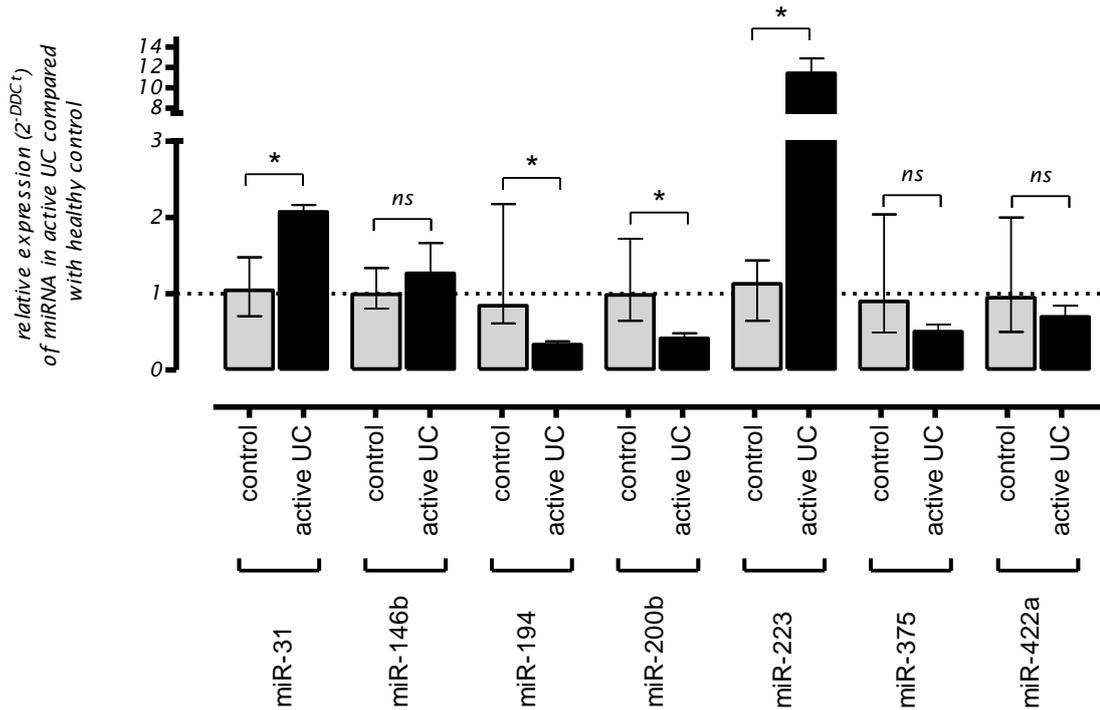


Figure 38. The relative expressions of candidate miRNAs in cells extracted from mucosa by Percoll centrifugation. The relative expression of each miRNA was analysed by RT-qPCR. The miRNA expressions are normalised to RNU44 and compared against healthy control. Median values are compared with a two tailed Mann Whitney U test. Whiskers = IQR, \*  $p < 0.05$ , ns = not significant. Probability statistics are shown in appendix 8.6.3.

The 4 miRNAs which have a decreased expression in whole mucosal biopsies also have a decreased expression in cell suspension. The expressions of miR-194 and miR-200b have a median fold decrease of 0.335 (IQR 0.091 – 0.380) and 0.415 (IQR 0.011 – 0.481) respectively. The decreased expressions of miR-194 and miR-200b are statistically significant,  $p = 0.014$  and  $p = 0.014$  respectively. The expressions of miR-375 and miR-422a are decreased in the cell suspension of patients with active UC compared with healthy controls but do not reach significance. Relative expressions of 0.505 (IQR 0.144 – 0.597,  $p = 0.100$ ) and 0.699 (IQR 0.231 – 0.845,  $p = 0.100$ ) respectively.

## 5.4 The expression of candidate microRNAs In CD4<sup>+</sup>CD25<sup>intermediate</sup> mucosal lymphocytes

The expression of miR-31 in umbilical cord blood CD4<sup>+</sup>CD25<sup>-</sup> cells is five times that of CD4<sup>+</sup>CD25<sup>+</sup>, and is thought to negatively regulate FoxP3 expression, at least another 9 miRNAs are also differentially expressed between CD25<sup>+</sup> and CD25<sup>-</sup> cells (Rouas et al., 2009). This gives rise to two important implications; the first is that miRNA expression is cell (and probably cell subtype) specific, the second is that miRNAs can influence lymphocyte cell development and / or function. We hypothesise that miRNAs play a crucial role in many inflammatory pathways. It is therefore helpful when looking at lymphocyte miRNA profiles to differentiate between pro-inflammatory and regulatory (T<sub>regs</sub>) CD4<sup>+</sup> cells.

Regulatory T cells are anti-inflammatory CD4<sup>+</sup> cells which are characterised by the expression of FoxP3. FoxP3 is an intracellular transcription factor and acts as a master regulator of T<sub>reg</sub> development and function. Disruption of the T<sub>reg</sub> pathway has been shown to predispose or worsen experimental inflammation and in humans T<sub>reg</sub> absence leads to IPEX syndrome of which colitis is a feature.

The process of intercellular staining for FoxP3 prohibits the analysis of miRNA. A widely used alternative to identify the T<sub>reg</sub> subfamily from the total CD4<sup>+</sup> pool is the presence of surface CD25. The marker CD25 is expressed on the surface of activated CD4<sup>+</sup> T cells. In the peripheral blood, a majority of CD4<sup>+</sup> cells that stain highly for CD25 (CD4<sup>+</sup>CD25<sup>high</sup>) express FoxP3 which is almost absent from CD4<sup>+</sup> cells expressing low levels of CD25 (CD4<sup>+</sup>CD25<sup>low</sup>) (Roncador et al., 2005, Eastaff-Leung et al., 2009). A method of reducing the FoxP3 population within a CD4<sup>+</sup> pool is to separate CD25<sup>low/intermediate</sup> from CD25<sup>high</sup>.

### 5.4.1 Flow cytometry

Lymphocytes within the cell suspensions are first identified by gating on an area of the dot plot which contained the highest density of cells, where lymphocytes are expected to be located and which demonstrated maximum CD3<sup>+</sup> fluorescence, Figure 39a. This population contained on average 32.2% (13.7 – 43.9) of all cells in the cell suspension, the rest presumably being other cells and cellular debris.

Within this population 54.2% (27.5 – 99.3%) stained for CD3, an example of CD3<sup>+</sup> staining characteristics are shown in Figure 39b.

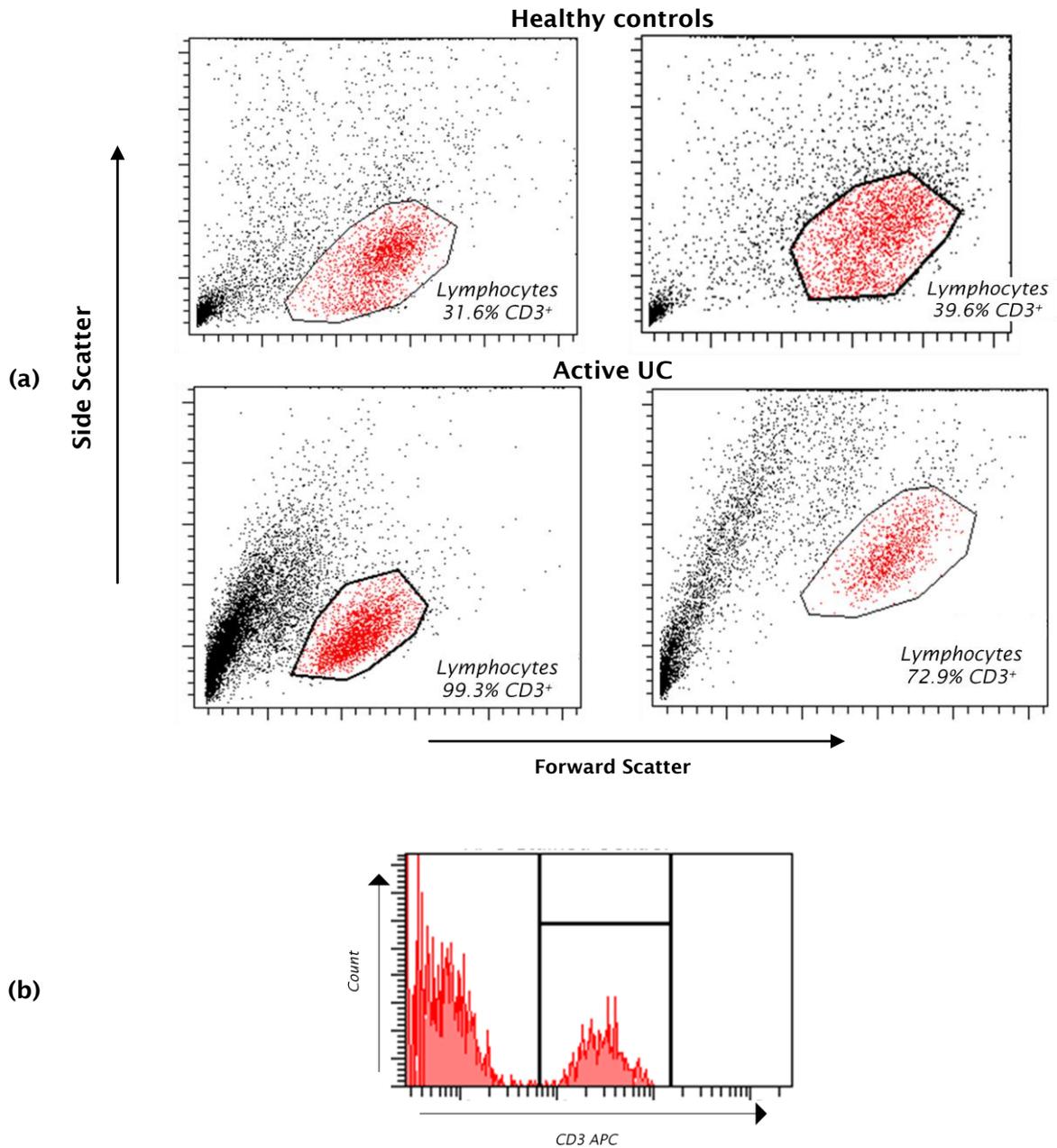


Figure 39. The initial stage of flow cytometry. (a) 2x Representative examples of a dot plots from healthy control and active UC. In each dot plot lymphocytes were separated by gating around populations of cells which contained as many of the CD3<sup>+</sup> cells as possible, enclosed populations. (b) An example of the CD3<sup>+</sup> signal from the gated population is illustrated in the histogram.

The lymphocyte population was assessed for CD3 and CD4 characteristics. For each sample a population of CD3<sup>+</sup>CD4<sup>+</sup> cells was clearly identified, Figure 40a. The mean number of CD3<sup>+</sup> lymphocytes per sample is 889 (range 293 – 2046) of these the mean number co-expressing CD4<sup>+</sup> is 397 (range 220 – 1695). Within the CD3<sup>+</sup> lymphocyte population a mean of 81% (range 75% – 84%) stained for CD4<sup>+</sup> there was no difference in this ratio between UC and control.

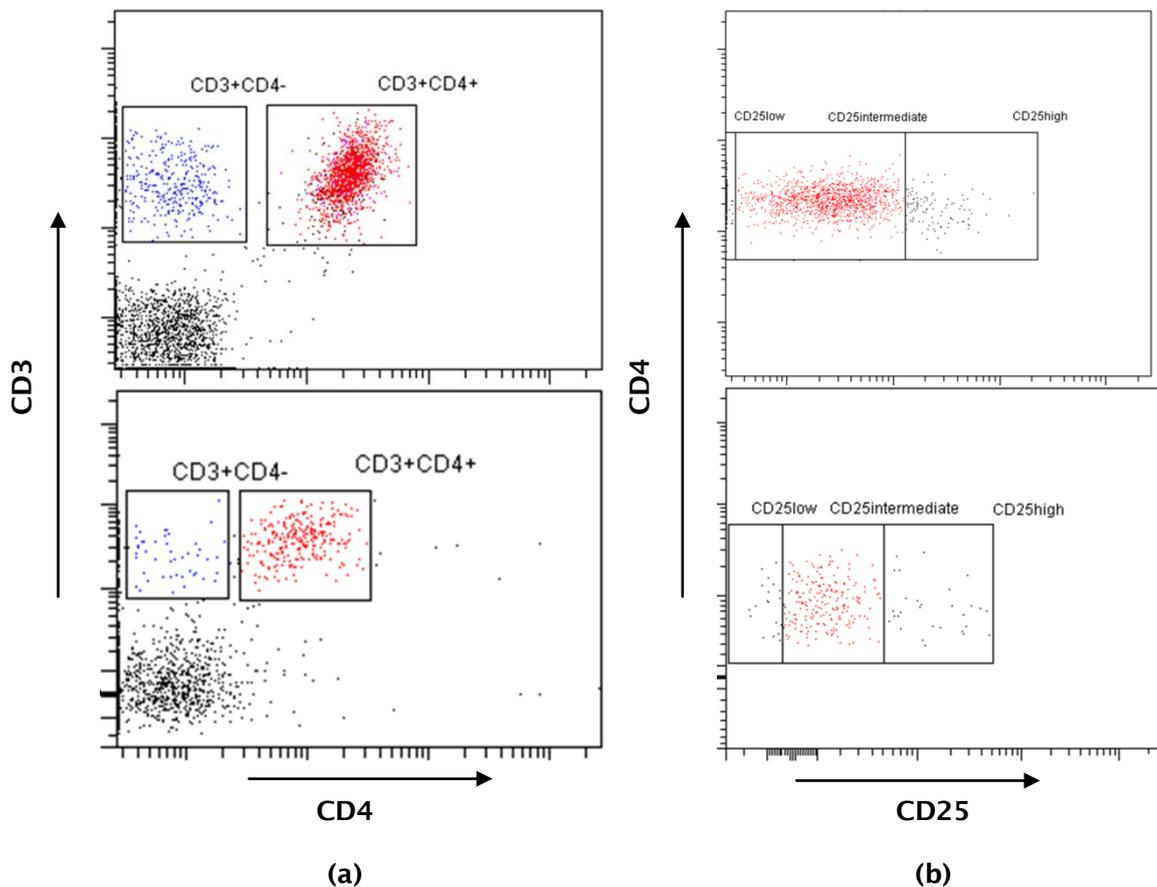


Figure 40. FACS for lymphocyte sub-populations. (a) Representative dot plot diagrams showing gating of lymphocytes into CD4<sup>+</sup> and CD4<sup>-</sup> subpopulations. (b) Representative dot plot diagrams of CD4 according to CD25 staining. CD3<sup>+</sup>CD4<sup>+</sup> cells are subdivided into CD25 staining characteristics, the percentage of the total CD3<sup>+</sup>CD4<sup>+</sup> population in low, intermediate and high CD25 staining are 10%, 70% and 20% respectively.

CD3<sup>+</sup>CD4<sup>+</sup> cells were further subdivided into CD25<sup>low</sup>, CD25<sup>intermediate</sup> and CD25<sup>high</sup>, with the proportions 10%, 70% and 20% respectively, Figure 40b. The mean number of



Figure 42. Relative expression of candidate miRNAs in CD4<sup>+</sup>CD25<sup>intermediate</sup> mucosal lymphocytes of active UC (n = 3) compared with healthy controls (n = 5). The expression of miRNAs was normalised to RNU44. Bars represent median values. Whiskers = IQR. Median values are compared with the one tailed Mann Whitney U test. \* = p ≤ 0.05. ns = not significant.

The percentage of FoxP3 within the CD4<sup>+</sup>CD25<sup>intermediate</sup> and the CD4<sup>+</sup>CD25<sup>high</sup> populations was analysed in 2 healthy controls and 1 active UC patient, due to the small cell numbers in the other samples. The mean percentage of CD4<sup>+</sup>CD25<sup>intermediate</sup> that stain for FoxP3<sup>+</sup> is 4% (range 0.0 – 9.0), compared with 45% (range 30.5– 70.4) in the CD4<sup>+</sup>CD25<sup>high</sup> population, Figure 41.

The miRNAs which are raised in the mucosa and mucosal lymphocytes of active UC are also all significantly increased in CD4<sup>+</sup>CD25<sup>intermediate</sup> mucosal lymphocytes.

The relative expressions of miR-31, miR-146b and miR-223 are increased by 3.689 (IQR 2.935 – 3.855, p = 0.018) fold, 155.8 (IQR 20.96 – 159.4, p = 0.029) fold and 6.343 (IQR 5.779 – 7.842, p = 0.018) fold respectively compared with healthy controls.

The expressions of miR-194, miR-200b, miR-375 and miR-422a are not significantly different from healthy controls.

## 5.5 Chapter discussion

The expression of 7 miRNAs has been shown to be abnormal in whole mucosal biopsies. The mucosa consists of a number of cell types which are likely to express different levels of miRNA depending on their function and local environment. Abnormal lymphocyte function is thought to be central to the pathogenesis of IBD with UC favouring a T<sub>H</sub>2 inflammatory response. Here we analyse the expression of these 7 miRNAs firstly in a cell suspension derived from mucosal biopsies by Percoll centrifugation, followed by the CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocyte subset.

The pattern of candidate miRNA expression is similar in the cell suspension, which consists of 32.2% lymphocytes, to that found in whole mucosal biopsies. The expressions of miR-31, miR-146b and miR-223 are increased in active UC and the expressions of miR-194, miR-200b, miR-375 and miR-422a are decreased. There are several differences between mucosa and cell suspension miRNA expression; the first is that the predominant feature is the 11.41 fold increase in miR-223 and second are that although increased and decreased respectively, the changes in miR-146b, miR-375 and miR-422a do not reach statistical significance in the cell suspension.

Can the results of the cell suspension analysis be interpreted as the expression profiles of “mucosal lymphocytes”? The literature would suggest that Percoll centrifugation will enrich a cell suspension extracted from human colon to 60 - 70% lymphocytes (Zhang et al., 2005). In this study, the cell suspension only yielded a viable CD3<sup>+</sup> lymphocyte population of 32.2% as measured by flow cytometry. This may be due to a number of factors including the composition of the cell solution loaded onto the gradient, pipetting, cell death prior to flow cytometry and / or other technical factors. The presence of other cell types in this suspension limits the interpretation with regards to “lymphocyte” miRNA expression. However this is a vastly enriched suspension because in health lymphocytes account for much < 5 % of the total number of cells in a mucosal biopsy. Overall, although the result should be interpreted with caution, cell

suspension analysis is a step closer to understanding the miRNA expression on specific cell types.

The CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocyte subset was considered for miRNA profiling as these are active pro-inflammatory T lymphocytes in UC. This subset was extracted initially on the basis of identifying a population of CD3 and CD4 staining cells, and then separated dependant on the strength of CD25 staining. Thus the population of naive CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>low</sup> mucosal lymphocytes was separated from activated CD25<sup>intermediate/high</sup> and discarded.

Activated CD4<sup>+</sup> lymphocytes consist of both regulatory and pro-inflammatory cells. These express both miRNAs and cytokines differently and so require separation. The regulatory cells express CD25 brightly resulting in a majority of regulatory T cells staining within the CD25<sup>high</sup> subset. The CD25<sup>high</sup> subset consisted of 45% FoxP3<sup>+</sup> cells compared with the CD25<sup>intermediate</sup> subset which is only 4% FoxP3<sup>+</sup>. To define low, intermediate and high staining CD25 subsets arbitrary proportions were chosen as there is no consistent division across the literature. By defining the CD25<sup>intermediate</sup> as only 4% FoxP3<sup>+</sup> regulatory T cells it is assumed that miRNA and cytokine profiles will be that of activated pro-inflammatory CD4<sup>+</sup> cells.

In CD4<sup>+</sup>CD25<sup>intermediate</sup> cells the expression of miR-31, miR-146b and miR-223 were increased with statistical significance. All three of these miRNAs are known to play a role in T cell function. Taken together the expression of miR-31 and miR-223 are consistently increased in the mucosa, cell suspension and CD4<sup>+</sup>CD25<sup>intermediate</sup> mucosal lymphocytes, in active UC.

There is a paucity of research on lymphocytes extracted from the colon of patients with UC who are not undergoing resection, or are not on immune altering medication. Lack of research is mainly due to the availability of appropriate volunteers and / or the difficulty in extracting lymphocytes from small quantities of tissue. Lymphocyte research to date has focused on experimental models or cell lines, research performed on human tissue has focused on lymphocytes extracted from peripheral blood, organs such as lymph nodes and spleen or lymphocytes extracted from surgical resection specimens. Although useful previous studies have relied on certain assumptions to extrapolate their results into human IBD,

## **Chapter 5. MicroRNA in mucosal lymphocytes**

**which may be one reason why their conclusions fail to translate well into clinical practice.**

**Together with the most consistently deregulated miRNA in UC, miR-31, the next step is to show the association between miR-31 and TSLP in UC and develop an in vitro model to analyse the relationship between miR-31 and TSLP in lymphocytes.**

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**Chapter 6. The association between miR-31  
and TSLP**

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## 6.1 Chapter abstract

The expression of miR-31 is consistently raised in the mucosa, mucosal lymphocytes and the CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocyte subset of active UC. Bioinformatic searches predict that TSLP is a target of miR-31 through the complementary base pairing of the seed region of miR-31 and the 3' UTR of TSLP.

Previous studies have failed to show the expression of TSLP in peripheral blood lymphocytes. Using RT-qPCR we confirm that peripheral blood mononuclear cells do not express TSLP, however stimulation with PHA produces a measurable expression of TSLP in a time dependent manner. We also show that lymphocytes extracted from the sigmoid mucosa constitutionally express TSLP and that the expression of TSLP in UC is decreased compared with healthy controls in mucosal biopsies, mucosal lymphocytes and CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocyte subset.

Using an in vitro lymphocyte cell model, the expression of miR-31 by PHA stimulated Jurkat cells is increased by over 500% with a strongly correlated decrease in TSLP mRNA expression of 28% compared with control, co-culture with the antagomir of miR-31 abrogates both the increase in miR-31 and the decrease in TSLP mRNA.

The effect of PHA stimulation on TSLP protein is greater than TSLP mRNA expression suggesting a post transcriptional effect. In stimulated Jurkat cells TSLP protein is decreased by ≈50% which is a twofold greater difference than that seen with the mRNA. In stimulated Jurkat cells co-cultured with the antagomir of miR-31 the expression of TSLP mRNA is the same as control.

Altered expressions of TSLP mRNA and protein in stimulated Jurkat cells are abrogated when co-cultured with the antagomir of miR-31 indicating that miR-31 plays a role in the TSLP pathway in lymphocytes.

## 6.2 Chapter introduction

Thymic stromal lymphopoietin (TSLP) is predicted to be a target for 4 of the 7 miRNAs that have been identified as having a dysregulated expression in UC. Thymic stromal lymphopoietin is constitutionally expressed by the epithelial cells (ECs) of the GI tract where the most well established actions are to directly alter DC function and the down-stream effects on T cells. In mice models it has also been well established that TSLP exerts an effect on T cells directly, although evidence for the direct effect of TSLP on T cells in humans is limited.

The influence of TSLP occurs through binding with the cell surface TSLP receptor. The receptor consists as a heterodimer of TSLPR and IL-7R. Once bound to its receptor, TSLP exerts its influence by activation of the STAT5, STAT3 (in mice) as well as other unknown pathways.

Epithelial cells and dendritic cells form the first line of defence against luminal antigens; under normal conditions expression of TSLP by ECs confers a non-inflammatory environment by promoting  $T_H2$  maturation and differentiation, IgA secretion by B cells, as well as other homeostatic functions such as maintenance of tight junction integrity and proliferation of regulatory T cells (Reche et al., 2001, Soumelis and Liu, 2004).

To date the predominant role for TSLP has been in the aetiology of allergic  $T_H2$  inflammatory conditions of the respiratory tract where it is seen to be increased in Asthma and rhinitis. There is no known role for TSLP in UC although it has been observed to be increased in surgically resected UC (Tanaka et al., 2010), and has a decreased expression in Crohn's where it is thought to play a role by the absence of the non-inflammatory influence on DCs (Rimoldi et al., 2005). It is the role of TSLP in  $T_H2$  mediated inflammation that is potentially interesting for UC.

Murine and human dendritic cells preferentially promote  $T_H2$  differentiation in response to oral antigens (Alpan et al., 2001) and during mucosal infection (Rimoldi et al., 2005). However DCs not exposed to the epithelium, monocyte derived DCs (MoDCs), do not favour  $T_H2$  differentiation over  $T_H1$  suggesting something in the mucosal environment drives a  $T_H2$  response (Rimoldi et al., 2005).

In response to invasion by bacteria and/or stimulation by various cytokines, epithelial cells increase their expression of TSLP and dendritic cells increase their expression of the TSLP receptor and IL-7R $\alpha$ . This is demonstrated by MoDCs which do not constitutionally express functional TSLP receptor but when exposed to the supernatants of caco-2 cells they express both IL-7R $\alpha$  and TSLPR (Rimoldi et al., 2005). Also culture of caco-2 cells with *s. typhimurium* increases the expression of TSLP by 2-3 fold, and culture of HT29 cells with IL-13 (a T<sub>H</sub>2 inflammatory cytokine) increases the expression of TSLP >8 fold (Biton et al., 2011).

Bacteria adhered to the basolateral membrane of the epithelial layer is indicative of more serious invasion. Under these circumstances a greater pro-inflammatory reaction with features of a T<sub>H</sub>1 response is seen, this suggests that the protective effect of a less aggressive T<sub>H</sub>2 response has been overcome. The loss of a TSLP influence indicates a narrow window of effect which may be reflected clinically by the undetectable levels of TSLP found in epithelial cells in 2/3 of patients with active Crohn's (Biton et al., 2011) and reduced levels seen in experimental colitis (Spadoni et al., 2012).

In humans, TSLP is expressed by epithelial cells which prime mucosal DCs to induce maturation and clonal proliferation of T cells, notably T<sub>regs</sub> as well as the release of T cell-attracting chemokines. In murine models, TSLP has additional features including expression by DCs independently of epithelial cells and the growth and differentiation of pre-B cells and peripheral CD4<sup>+</sup> T cells including the development of T<sub>regs</sub> (Spadoni et al., 2012) independently of DCs. Although largely confined to murine models TSLP may also directly influence T cell function. In humans activated peripheral CD4<sup>+</sup> lymphocytes show an expression of TSLP receptor that is not seen in naive CD4<sup>+</sup> cells and when activated CD4<sup>+</sup> cells are co-cultured with TSLP they show increased proliferation and sensitivity to IL-2 (Rochman et al., 2007). Differences in TSLP function between murine and human disease may in part be because they share only 43% amino acid homology, this must be taken into account when interpreting and applying experimental models into human results.

## Chapter 6. The association between miR-31 and TSLP

To understand the role of TSLP in UC, the first step is to quantify the expression of TSLP in active UC, akin to other  $T_H2$  inflammatory conditions we would expect the expression of TSLP to be increased. We have already shown the differential levels of miR-31 in active mucosa and mucosal lymphocytes, to study the relationship between miR-31 and TSLP further an in vitro model of stimulated lymphocytes is developed to simulate mucosal lymphocytes.

Previously the expression of TSLP has been thought to be largely from epithelial cells, this has been driven by the research on TSLP involving skin and respiratory pathologies, recent reviews however have shed doubt on this assumption and revealed that TSLP is expressed by numerous cell types (Takai, 2012). Despite the TSLP being previously thought of as an epithelial derived factor, the increased expression of miR-31 in mucosal  $CD4^+CD25^{\text{intermediate}}$  lymphocytes, and the targeting of TSLP by miR-31 has focused this chapter on TSLP expression by lymphocytes specifically.

Using an in vitro cell model in which Jurkat cells are co-cultured with PHA and an antagomir to miR-31 it is demonstrated that the expression of TSLP mRNA and TSLP protein are influenced by the manipulation of miR-31 expression.

Manipulation of miRNA expression can be achieved through specific miRNA genetic alteration, generally through alterations of the miRNA biosynthesis pathway, or through miRNA post transcriptional repression. In this study miR-31 expression is manipulated through the transfection of cells with the antagomir of miR-31. Antagomirs exert their effect through the base pair complementarity and binding with specific miRNA which prevents integration of the miRNA into the RISC complex, thus silencing its function. The antagomir / miR-31 molecule is eventually degraded by the normal cellular processes.

### 6.3 The expression of TSLP mRNA is decreased in active UC

Ulcerative Colitis is still considered a T<sub>H</sub>2 type inflammatory response, so in line with other T<sub>H</sub>2 inflammatory conditions it is expected that TSLP has an increased expression in the mucosa of active UC. However, the success of various TNF- $\alpha$  antagonists in practice points towards a less polarised aetiology. We hypothesize that post transcriptional regulation of TSLP by miR-31 decreases the expression of TSLP in UC which may lead to a response which could favour T<sub>H</sub>1 characteristics.

#### 6.3.1 TSLP mRNA expression is decreased in the sigmoid mucosa

		Control (n = 8)	Inactive UC (n = 8)	(U value, p value)	Active UC (n = 8)	(U value, p value)
Demographics	Age	62 (45 - 76)	59 (51 - 66)	25, p = 0.495	52 (29 - 63)	22.5, p = 0.342
	Sex (F/M)	4/4	3/5		3/5	
	Ethnicity (if not white)					
	Smoker / Ex Smoker <sup>1</sup>		1x smoker 1x ex smoker <sup>1</sup>		3x ex smokers <sup>1</sup>	
Disease	Months since diagnosis	0 (0 - 0)	165 (127 - 295)		69 (7 - 1271)	
	Concomitant drugs	8 x nil	8 x nil		8 x nil	
	disease extent (maximal/today)					
	normal	8/8	0/8		0/0	
	Left sided	0/0	6/0		5/4	
total	0/0	2/0		3/4		
Clinical Scores	Baron score	0 (0 - 0)	0 (0 - 0)		3 (3 - 3)	
	Mayo Score	0.0 (0.0 - 4.5)	0.0 (0.0 - 0.0)		8.6 (6.0 - 11.5)	
	Truelove and Witts	8x normal	8x normal		2x mild 3x moderate 3x severe	
Clinical investigation	Deviation on Hct (%)	2.3 (0.7 - 6.6)	0.0 (0.0 - 1.6)	9.5, p = 0.058	6.0 (2.9 - 8.2)	26.5, p = 0.591
	Weight loss (%)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	28, p = 1.000	0.0 (0.0 - 4.8)	24, p = 0.467
	Temperature >37.5°C	nil	nil		nil	
	Pulse >90 bpm	nil	nil		nil	
	Hb	130 (128 - 145)	142 (141 - 142)	4.5, p = 0.583	135 (113 - 147)	26.5, p = 0.888
	ESR	9 (5 - 27)	15 (8 - 21)	4.5, p = 1.000	36 (13 - 60)	4, p = 0.167
	CRP	2 (2 - 5)	6 (4 - 7)	1.5, p = 0.238	15 (7 - 34)	3, p = 0.018
	Raised Creatinine <sup>2</sup>	nil	nil		nil	
Albumin	41 (37 - 45)	43 (40 - 45)	5, p = 0.694	38 (36 - 42)	19, p = 0.321	

Table 43. Patient characteristics for the mucosal biopsies used to examine TSLP mRNA expression in UC. Values displayed as medians (IQR), <sup>1</sup>has not smoked within 3 months of biopsy, <sup>2</sup>above laboratory reference range. Statistical relevance of the difference between the median values and the control group are calculated using the two tailed Mann Whitney U test, probability statistics are shown as the (U value, p value).

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Samples are matched for age, sex and ethnicity, Table 43. All patients in the active UC group had Baron grade 3 macroscopic inflammation. In the active UC group, clinical severity as measured by the Truelove and Witts criteria varied from mild to severe. Clinical measures of disease activity as measured by deviation of Hct, percentage weight loss, Hb and albumin showed no difference between healthy control and active UC. Although CRP was significantly higher in the active UC group compared with healthy control,  $p = 0.018$ , there was no significant difference in the level of ESR.

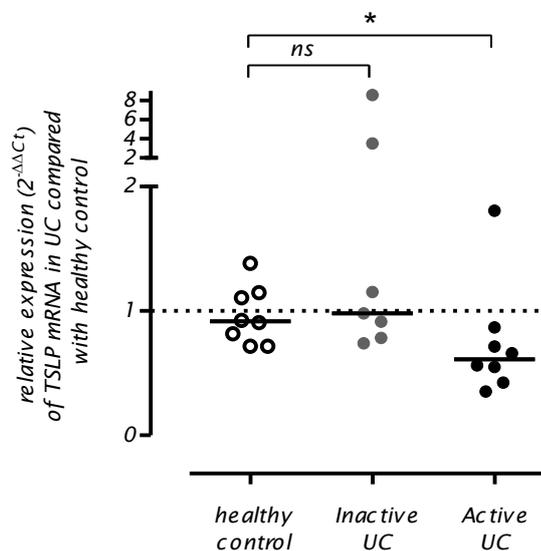


Figure 43. Relative expression of TSLP mRNA in the sigmoid mucosa. The expression of TSLP mRNA in active and inactive sigmoid UC is compared against healthy controls using TaqMan™ probe based RT-qPCR. TSLP mRNA expression is normalised to GAPDH and compared against healthy controls. Median values are compared using a one tailed Mann Whitney U test. \*  $p < 0.05$ , ns = not significant.

The expression of TSLP mRNA is decreased by 0.612 (IQR 0.456 – 0.830,  $p = 0.014$ ) fold in active sigmoid UC compared with healthy control.

There is no significant difference in the expression of TSLP in the sigmoid mucosa of inactive UC compared with healthy controls,  $p = 0.198$ .

### 6.3.2 TSLP protein is decreased in the sigmoid mucosa of active and inactive UC<sup>11</sup>

		Control (n = 12)	Inactive UC (n = 12)	(U value, p value)	Active UC (n = 11)	(U value, p value)
Demographics	Age	58 (43 - 70)	55 (48 - 67)	28, p = 0.342	49 (29 - 63)	24, p = 0.167
	Sex (F/M)	6/6	6/6		5/6	
	Ethnicity (if not white)					
	Smoker / Ex Smoker <sup>1</sup>		3x ex smoker <sup>1</sup>		2x ex smokers <sup>1</sup>	
Disease	Months since diagnosis	0 (0 - 0)	210 (97 - 310)		72 (8 - 1123)	
	Concomitant drugs	12 x nil	12 x nil		11 x nil	
	disease extent (maximal/today)					
	normal	12/12	0/12		0/0	
	Left sided total	0/0	4/0		6/5	
Clinical Scores	Baron score	0 (0 - 0)	0 (0 - 0)		3 (3 - 3)	
	Mayo Score	0.0 (0.0 - 5)	0.0 (0.0 - 0.0)		9.0 (6.0 - 12.5)	
	Truelove and Witts	12x normal	12x normal		2x mild 5x moderate 4x severe	
Clinical investigation	Deviation on Hct (%)	4.0 (0.5 - 5.5)	0.0 (0.0 - 2.0)	10, p = 0.167	8.0 (3.2 - 10.0)	20, p = 0.467
	Weight loss (%)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	20, p = 1.000	0.0 (0.0 - 6.0)	24, p = 0.342
	Temperature >37.5°C	nil	nil		nil	
	Pulse >90 bpm	nil	nil		nil	
	Hb	129 (125 - 144)	145 (138 - 143)	6, p = 0.467	136 (110 - 145)	24, p = 0.888
	ESR	6 (3 - 15)	18 (4 - 15)	4, p = 0.888	40 (15 - 58)	4, p = 0.018
	CRP	3 (0 - 11)	5 (2 - 8)	3, p = 0.167	19 (8 - 33)	3, p = 0.018
	Raised Creatinine <sup>2</sup>	nil	nil		nil	
Albumin	40 (36 - 43)	42 (38 - 44)	6, p = 0.467	37 (33 - 43)	12, p = 0.281	

Table 44. Patient characteristics for the mucosal biopsies used to examine TSLP protein expression in UC. Values displayed as medians (IQR), <sup>1</sup>has not smoked within 3 months of biopsy, <sup>2</sup>above laboratory reference range. Statistical relevance of the difference between the median values and the control group are calculated using the two tailed Mann Whitney U test, probability statistics are shown as the (U value, p value).

Samples were collected and protein concentration analysed with ELISA as described in Chapter 2. Patients were matched for age and sex. All patients are treatment naive and patients in the active UC group all have Baron grade 3 macroscopic inflammation.

<sup>11</sup> The sample collection, experiment and analysis for section 6.3.2 was performed by Dr Simon Whiteoak and reproduced with permission

## Chapter 6. The association between miR-31 and TSLP

Patient characteristics are outlined in Table 44. There is a significant difference in the ESR and CRP between the active UC group and healthy controls in keeping with an inflammatory process. In the inactive UC group there is no significant difference in ESR and CRP indicating the absence of inflammation.

In all three groups the protein concentration was low. In healthy controls the median 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 (IQR 1.4 - 5.5).

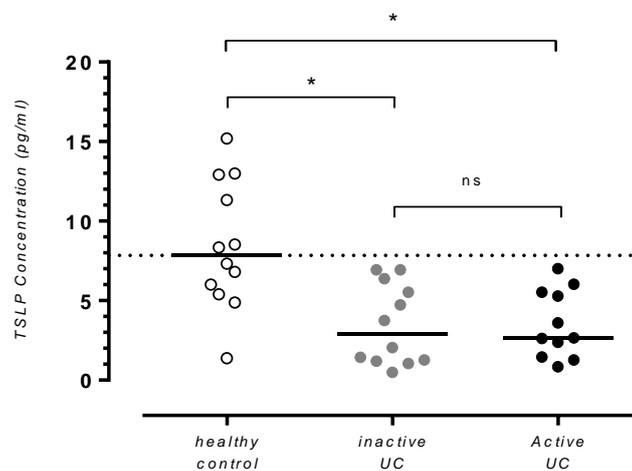


Figure 44. TSLP protein concentration in active and inactive UC. Mucosal biopsies are homogenised and protein concentration analysed in the supernatant by ELISA. TSLP concentration was measured in healthy controls (n = 12), inactive UC (n = 12) and active UC (n = 11). Dotted line shows the protein expression in the control group. Median values are compared with the two tailed Mann Whitney U test. \* p < 0.05, ns = not significant. Experiment and analysis with ELISA performed by Dr Simon Whiteoak.

The protein concentration in inactive UC is 37% (p = 0.002) and in active UC 34% (p = 0.003) of that in healthy controls.

### 6.3.3 TSLP mRNA expression is decreased in mucosal lymphocytes

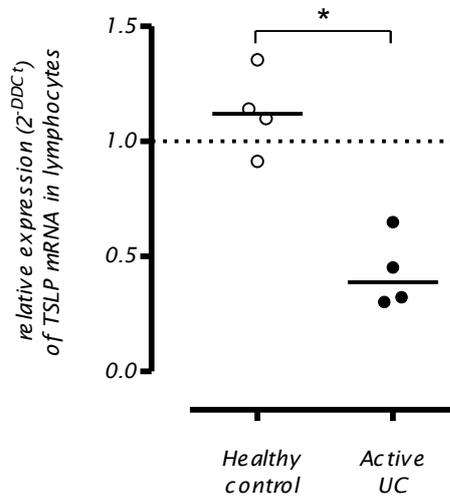


Figure 45. The expression of TSLP mRNA in mucosal lymphocytes. Mucosal lymphocytes were extracted using EDTA, collagenase and a Percoll® gradient. Messenger RNA was isolated and analysed with RT-qPCR. The relative expression of TSLP mRNA was normalised to GAPDH and compared against healthy controls, \* p = <0.05.

There is expression of TSLP by lymphocytes extracted from healthy mucosa. The expression of TSLP mRNA is decreased by 0.388 (IQR 0.308 – 0.600, p = 0.014) fold in mucosal lymphocytes extracted from the mucosa in active sigmoid UC compared with healthy controls.

### 6.3.4 TSLP mRNA expression is decreased in CD4<sup>+</sup>CD25<sup>intermediate</sup> mucosal lymphocytes

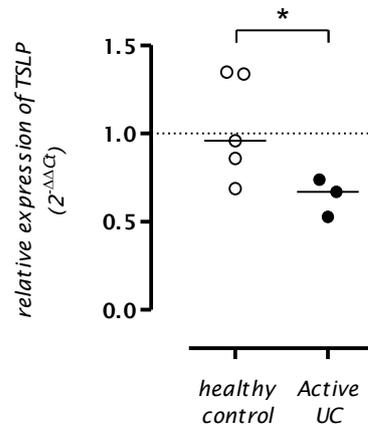


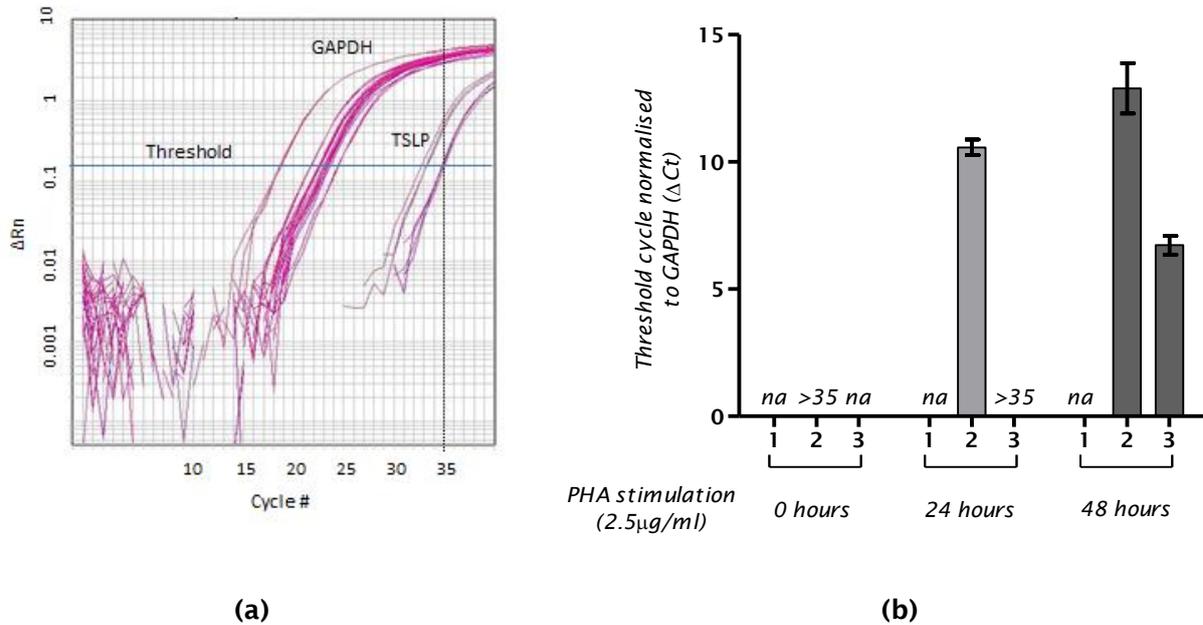
Figure 46. The expression of TSLP in CD4<sup>+</sup>CD25<sup>intermediate</sup> mucosal lymphocytes. The relative expression of TSLP mRNA in active UC (n = 3) is first normalised to GAPDH and compared with healthy controls (n = 5). Median values are compared using the one tailed Mann Whitney U test. \* = p ≤ 0.05.

In active UC the expression of TSLP mRNA in mucosal CD4<sup>+</sup>CD25<sup>intermediate</sup> is 0.669 (IQR 0.526 – 0.737, p = 0.036) fold relative to healthy controls.

### 6.3.5 TSLP expression in healthy PBMCs

Healthy peripheral PBMCs do not express TSLP mRNA. There is a measurable amplification of TSLP mRNA in 33% (1/3) of samples although the Ct value is above the threshold cycle considered significant. Peripheral PBMCs activated ex-vivo by co-culture with PHA show amplification of TSLP mRNA in 33% (1/3) of samples after 24 hours and in 66% (2/3) samples after 48 hours culture, Figure 47.

In 3 of the 9 wells there is measurable amplification of TSLP mRNA during RT-qPCR. In another 2 wells there is measurable TSLP but with Ct value above that considered significant.



(a) (b)

Figure 47. The expression of TSLP mRNA in healthy PBMCs. PBMCs from 3 healthy controls were extracted from the peripheral blood using Ficoll®. The PBMCs were divided into 3 and stimulated with PHA (2.5  $\mu g/ml$ ) for 0, 24 and 48 hours. RT-qPCR was performed. (a) The amplification plot of TSLP mRNA and GAPDH. (b) Comparison of  $\Delta Ct$  values for TSLP mRNA amplification in relation to the duration of stimulation by PHA. >35 = TSLP mRNA amplified but with a Ct value greater than 35.

In sample #2, there is a measurable quantity of TSLP mRNA in healthy PBMCs, but at 0 hours this is above the cut off Ct value. At 24 and 48 hours of co-culture with PHA the amplification is significant. In sample #3 there is no amplification at 0 hours but after 24 hours the amplification becomes measurable, but not significant, after 48 hours the amplification is measurable, Figure 47b.

## 6.4 Stimulation of Jurkat cells with PHA as a mucosal lymphocyte model for Ulcerative Colitis.

The expression of miR-31 is raised in whole mucosal biopsies, lymphocytes and CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocytes extracted from active UC. A target of miR-31 is predicted to be TSLP. The expression of TSLP has been shown to be decreased in mucosal biopsies, lymphocytes and CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocytes extracted from the sigmoid mucosa of active UC. Little is known of the expression and function of TSLP in UC and the expression of TSLP has not been previously reported in mucosal lymphocytes. The expression of TSLP has been shown to be raised in other T<sub>H</sub>2 inflammatory disorders where it is expressed by epithelial cells as a factor that influences dendritic and lymphocyte cell function.

The aim of this section is to develop an in-vitro model of colitis that mimics the miR-31 and TSLP mRNA expressions previously seen in mucosal samples.

Three distinct cell types are analysed for their expression of miR-31 and TSLP. Jurkat, THP-1 and HT29 cells are immortalised clones of human lymphocytes, monocytes and epithelial cells respectively. They represent three distinct cell types which are found within the colonic mucosa and have been implicated in the aetiology of UC.

### 6.4.1 Optimal culture conditions, the stimulation of PBMCs with PHA

Peripheral blood was taken from 3 healthy volunteers, 2 male and 1 female ages 32, 32, and 35 years. All are on no medication and have no current or previous medical history. The PBMCs were stimulated with PHA at 0.0 µg/ml, 2.5 µg/ml, 5.0 µg/ml and 10.0 µg/ml for 0, 24 and 48 hours as described in section 2.9.5.

The relative expression of miR-31 compared with unstimulated PBMCs increases over time for all concentrations of PHA. The relative expression of miR-31 appears to reach a peak at 48 hours with no further significant increase beyond this.

After 48 hours culture with PHA the expression of miR-31 in healthy PBMCs is increased by a median of 3.099 (IQR 2.666 – 4.134) fold, 3.247 (IQR 2.776 – 4.747)

fold and 2.212 (2.093 – 3.744) fold in cultures with 2.5 µg/ml, 5.0 µg/ml and 10.0 µg/ml PHA respectively. The relative expression of miR-31 is significantly increased at 48 hours between PBMCs not cultured with PHA and those cultured with 2.5 µg/ml PHA,  $p = 0.050$ . However the small increase of 3.099 to 3.247 between 2.5 µg/ml and 5.0 µg/ml respectively is not significant.

These results would indicate that the optimum culture conditions for the expression of miR-31 in PBMCs is 2.5 µg/ml PHA for 48 hours.

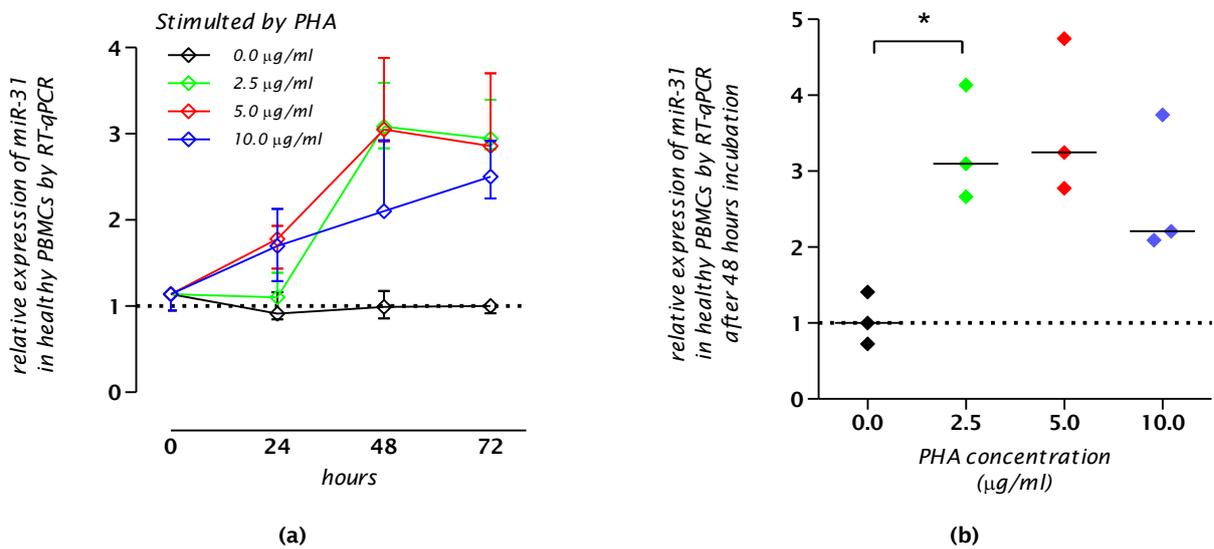


Figure 48. The expression of miR-31 in healthy PBMCs following culture with PHA. The expression of miR-31 is analysed in PBMCs of 3 healthy volunteers by RT-qPCR. The expression of miR-31 was normalised to RNU44 and compared against paired samples without PHA stimulation. (a) The expression of miR-31 increases over time for all concentrations of PHA, however there is no significant increase in miR-31 expression beyond 48 hours. (b) The relative expression of miR-31 at 48 hours. There is a significant difference in the expression of miR-31 at 48 hours after co-culture with 2.5 µg/ml PHA. Median values are compared using the one tailed Mann Whitney U test. \*  $p \leq 0.05$ .

### 6.4.2 Candidate miRNAs in Jurkat cells

The expressions of miR-31, miR-194, miR-223 and miR-422a are increased in Jurkat cells when cultured with 2.5 µg/ml PHA for 48 hours compared with control.

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The expressions of miR-31 and miR-223 in stimulated Jurkat cells mimic that seen in UC. Despite the expressions of miR-194 and miR-422a being increased in stimulated Jurkat cells, this is opposite to that found in UC suggesting that Jurkat cell stimulation with PHA is not a good model to mimic miR-194 or mir422 expression and their down-stream influence on gene transcription.

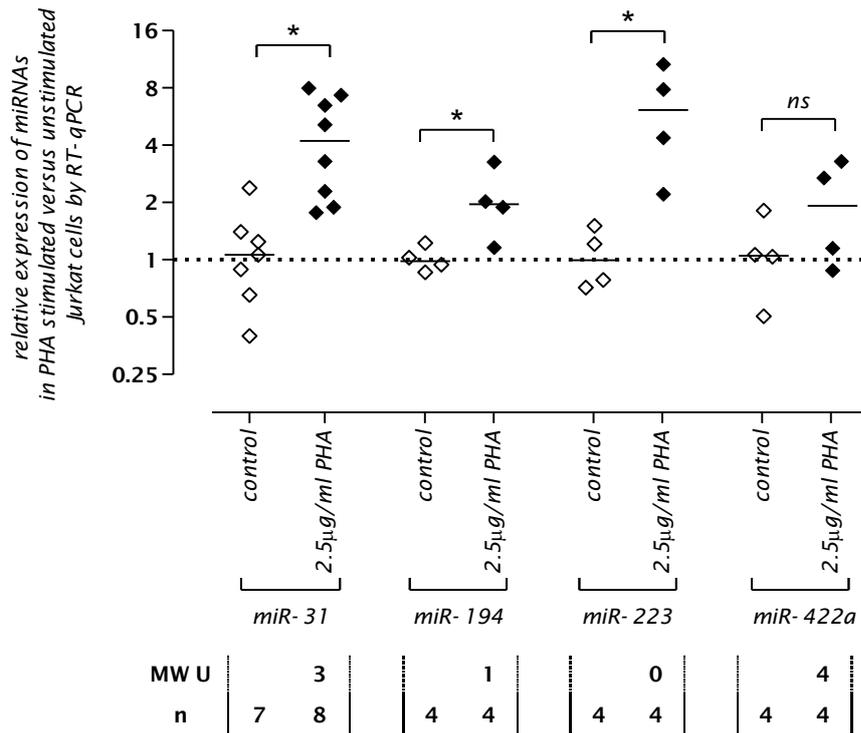


Figure 49. The relative expression of Candidate miRNAs in PHA stimulated Jurkat cells. Jurkat cells were cultured for 48 hours with, or without, 2.5 µg/ml PHA, and a RT-qPCR performed. The expressions of miRNAs were normalised to RNU44, and the expressions of miRNAs were compared in stimulated versus unstimulated (control) Jurkat cells. Median values were compared using the one tailed Mann Whitney U test. \* p ≤ 0.05. ns = not significant.

The expression of miR-194 is 1.959 (IQR 0.881 – 1.175, p = 0.029) fold greater in stimulated Jurkat cells compared with control. The expression of miR-422a is 1.924 (IQR 0.947 – 3.148) fold greater in stimulated Jurkat cells compared with control, although does not reach significance, p = 0.171.

The expression of miR-31 is 4.209 (IQR 1.993 – 7.110,  $p = 0.001$ ) fold greater in stimulated Jurkat cells compared with control. The expression of miR-223 is 6.122 (IQR 2.756 – 9.965,  $p = 0.014$ ) fold greater in stimulated Jurkat cells compared with control.

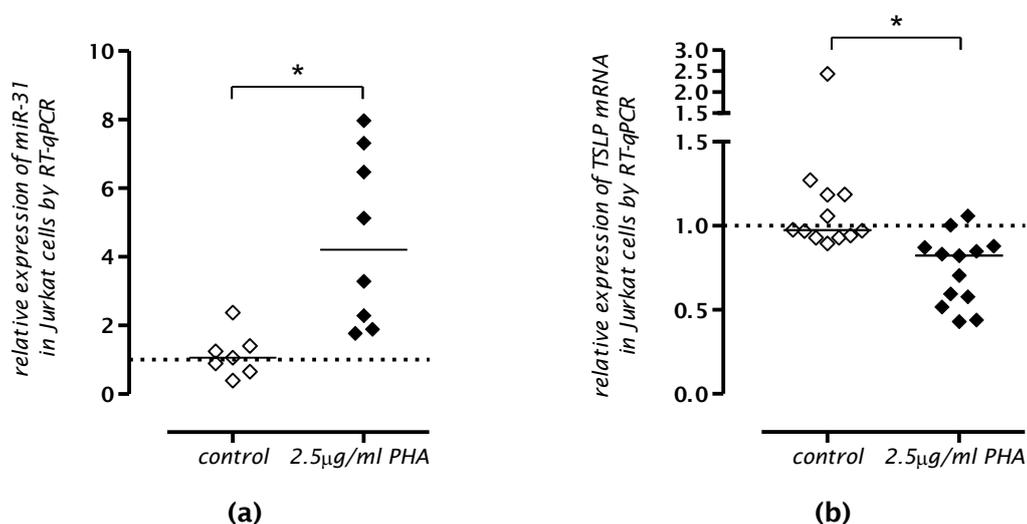
Of the 4 miRNAs which are predicted to have TSLP as a target, the expressions of miR-31 and miR-223 mimic the expressions in UC.

### 6.4.3 The association between miR-31 and TSLP

Both miR-31 and miR-223 have an increased expression in stimulated Jurkat cells and both are predicted to target TSLP. The focus of this section is miR-31 due to the greater expression of miR-31 in mucosal biopsies compared with miR-223, 10.970 versus 3.454 respectively, section 4.4.1. As a target of miR-31 it is expected that the expression of TSLP is reduced in stimulated Jurkat cells. In this section the association between miR-31 and TSLP in paired Jurkat cell samples is demonstrated.

The relative expressions of both miR-31 and TSLP follow the same pattern in stimulated Jurkat cells as that seen in whole mucosal biopsies, mucosal lymphocytes and CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocytes in UC, Figure 50a and b.

The expression of miR-31 is increased by 4.209 fold in stimulated Jurkat cells. The expression of TSLP mRNA is 0.822 (IQR 0.549 – 0.875,  $p < 0.001$ ) fold less in stimulated compared with unstimulated Jurkat cells.



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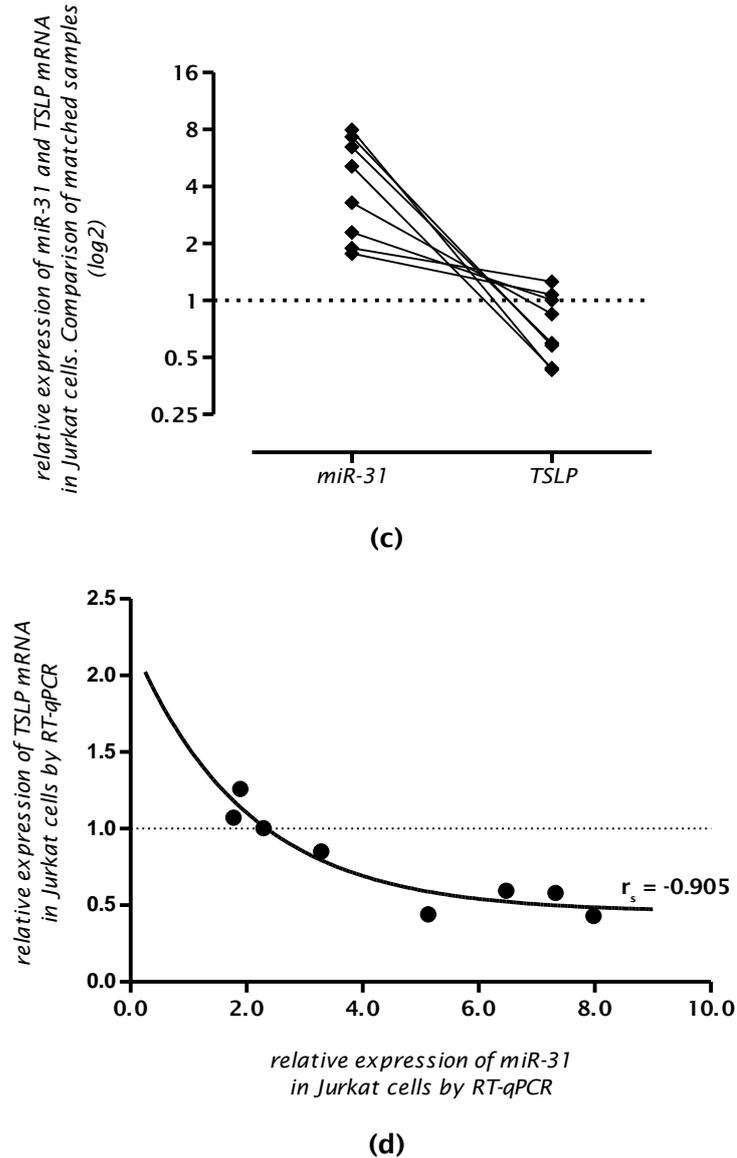


Figure 50. The association of miR-31 and TSLP in PHA stimulated Jurkat cells. The expression of miR-31 and TSLP mRNA are analysed using RT-qPCR. (a) The expression of miR-31 in PHA stimulated Jurkat cells ( $n = 8$ ) is normalised to RNU44 and compared against unstimulated Jurkat cells ( $n = 7$ ). (b) The expression of TSLP mRNA in PHA stimulated Jurkat cells ( $n = 13$ ) is normalised to GAPDH and compared against unstimulated Jurkat cells ( $n = 12$ ). For both miR-31 and TSLP there was a significant difference between the median values, MW  $U = 3$  and  $15$  respectively. (c) Comparison of the relative expression of miR-31 and TSLP in paired samples of stimulated Jurkat cells. (d) The association between the relative expression of miR-31 and TSLP in stimulated Jurkat cells. Best fit line calculated using one phase decay equation,  $[Y=(Y_0-\text{plateau})x(-KxX)+\text{plateau}]$ , (GraphPad Prism v5.04). Spearman's rank correlation coefficient  $r_s = -0.905$ ,  $p = 0.002$ .

The relationships between miR-31 and TSLP mRNA in paired samples are shown in Figure 50c. There is a significant inverse relationship between the expression of miR-31 and TSLP mRNA,  $r_s(6) = -0.905$ ,  $p = 0.002$ , Figure 50d. It appears that the influence of miR-31 on TSLP expression has a finite effect. The maximum inhibitory effect of miR-31 peaks when it's expression is between 8 and 10 times normal, an expression which halves the expression of TSLP.

## 6.5 Antagomir silencing of miR-31 blocks the effect of PHA on TSLP mRNA

We have shown that in human samples the expression of miR-31 is increased and the expression of TSLP mRNA is decreased in the mucosa, mucosal lymphocytes and CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocyte subset in active UC. We have also shown the same inverse relationship in stimulated Jurkat cells.

Using PHA stimulated Jurkat cells as an in vitro model to mimic the relationship between miR-31 and TSLP in lymphocytes in actively inflamed UC; we aim to show an association between the expression of miR-31 and TSLP mRNA and TSLP protein by silencing miR-31.

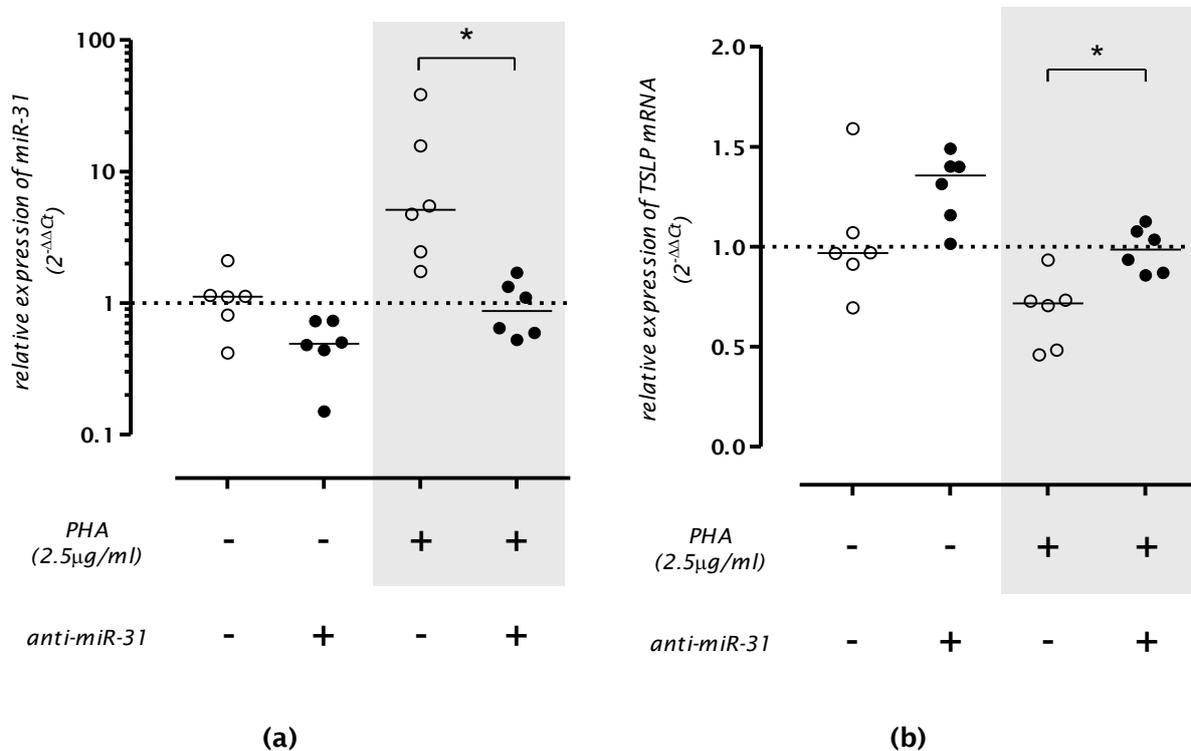


Figure 51. The relative expression of (a) miR-31 and (b) TSLP in stimulated Jurkat cells co-cultured with the antagomir of miR-31. Jurkat cells were co-cultured with or without PHA and either the antagomir to miR-31 or a scrambled sequence antagomir for control. Results are presented relative to the expression in unstimulated cells transfected with scrambled antagomir. All groups n = 6. Medians are compared with the one tailed Mann Whitney U test, \* P<0.05. Grey area = stimulated cells.

Specific silencing of miR-31 decreases the endogenous expression of miR-31 by  $\approx 50\%$  (relative expressions 1.117 vs. 0.490). As previously demonstrated stimulation of Jurkat cells increases the relative expression of miR-31, in this series of experiments the relative expression of miR-31 was 5.106 (IQR 2.277 – 21.390,  $p = 0.002$ ) fold compared with unstimulated cells. This increase is abrogated when stimulated cells are also cultured with miR-31 specific antagomir, 0.873 (IQR 0.577 – 1.421) fold, a difference which is significant,  $p = 0.001$ , Figure 51a.

In these same cultures there is an inverse relationship between miR-31 and TSLP. The endogenous expression of TSLP mRNA is increased by 1.357 fold when cells are cultured with miR-31 specific antagomir, although this does not reach significance  $p = 0.131$ .

In Jurkat cells stimulated with PHA, the relative expression of TSLP is 0.717 (IQR 0.477 – 0.783,  $p = 0.021$ ) fold compared with unstimulated cells. This difference is abrogated when stimulated cells are cultured with miR-31 specific antagomir, relative expression of TSLP is 0.985 (IQR 0.866 – 1.089) fold, a difference which is significant,  $p = 0.004$ , Figure 51b.

## 6.6 Silencing of miR-31 blocks the effect of PHA on TSLP protein

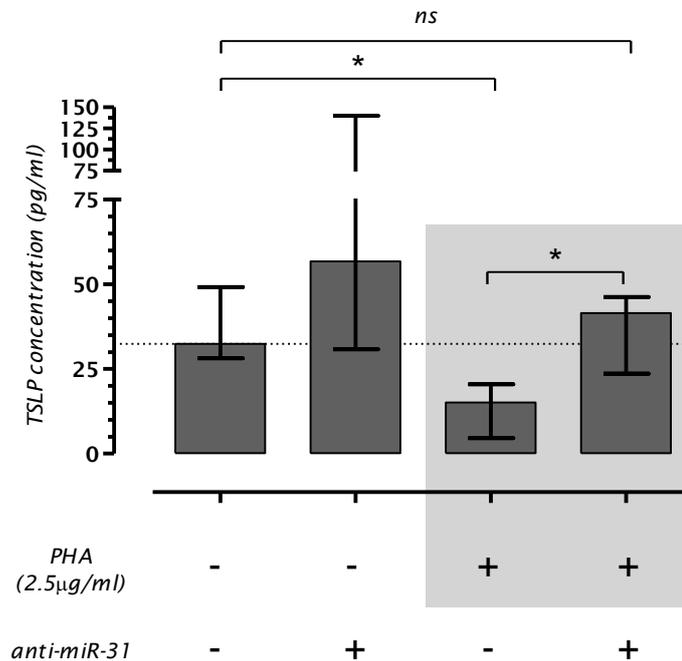


Figure 52. TSLP concentration in cell culture supernatants from Jurkat cells transfected with the antagomir to miR-31. Jurkat cells were co-cultured with or without PHA and either the antagomir to miR-31 or a scrambled sequence antagomir for control. Protein quantification was performed by ELISA. All groups n = 6. Dotted line shows the protein expression in the control group. Medians are compared with the one tailed Mann Whitney U test, \* P<0.05. Whiskers indicate IQR. Grey area = stimulated cells.

Stimulation of Jurkat cells with PHA decreases the concentration of TSLP by 53.4%,  $p = 0.001$ . In unstimulated cells the concentration of TSLP is 32.5 pg/ml (IQR 28.1 – 49.1) and in stimulated cells 15.1 pg/ml (IQR 4.6 – 20.5). Both cell cultures were co cultured with a scrambled sequence antagomir.

In cells stimulated with PHA the concentration of TSLP is 15.1 pg/ml, when co-cultured with miR-31 antagomir the concentration of TSLP is 41.5 pg/ml (IQR 23.6 – 46.3) a significant difference,  $p = 0.002$ .

This indicates that co-culture with the miR-31 antagomir abrogates the negative influence of PHA on TSLP expression. Interestingly the expression of TSLP in unstimulated cells is increased to 56.8 pg/ml (IQR 30.8 – 140.0) if co-cultured with

**the miR-31 antagomir, indicating that in the absence of miR-31 the basal expression of TSLP is greater.**

## 6.7 Chapter discussion

Although identified as being expressed in the healthy intestine in 2000 and absent from active Crohn's colon in 2005, it was not until a study by Tanaka et al. (2010) that the differential expression of TSLP was reported in the colonic mucosa of active UC. The finding of an increased expression of TSLP is consistent with the well documented increase in TSLP expression found in other  $T_H2$  mediated inflammatory conditions such as allergic respiratory and skin disorders.

In health there are several drivers for TSLP expression including infection and response to cytokines. During infection, TSLP has 2 roles, the first is to enhance pathogen expulsion, a feature particularly important in helminth infection, and the second role is to promote a less destructive inflammatory response and maintain epithelial barrier homeostasis through the promotion of  $T_H2$  cytokines including IL-4.

Cytokines which drive TSLP expression include IL-4, suggesting a positive feedback mechanism, IL-13 and IL-25 and the  $T_H1$  cytokines TNF- $\alpha$ , IL-1 and INF- $\gamma$ , suggesting an attempt to steer the inflammatory milieu away from a  $T_H1$  response. It is not known if TSLP triggers inflammation through the promotion of an exaggerated  $T_H2$  inflammation or is triggered during an inflammatory response as a control mechanism against a far more destructive  $T_H1$  response.

We found that the expression of TSLP mRNA and TSLP protein is decreased in whole biopsies taken during endoscopy from active sigmoid UC. This is opposite to the increase found in UC by Tanaka et al. (2012) and opposite to what is expected if UC is thought to parallel inflammatory respiratory conditions.

There are a number of features of the study by Tanaka et al. (2010) which make direct comparison challenging. Firstly, the study by Tanaka et al. includes a small and distinct population of Asian UC patients undergoing resection presumably for treatment resistant severe UC under which circumstances it is likely that they were also on high dose steroids as well as other immune altering medications. It is not stated from where the tissue is taken, and the comparison is not with healthy individuals as claimed but patients undergoing colorectal cancer resection. These

are all important factors given that; 1. There are important aetiological differences between Asian and Western IBD patients, not least genetic and environmental risk factors, 2. The annual incidence of colectomy is <1%, indicating that the study was performed on a highly selected and unique cohort from within the UC family, their immunological milieu is likely to have been altered by treatment including an effect on cytokines that influence TSLP expression, e.g. TNF- $\alpha$ , 3. There are regional differences in TSLP expression throughout the intestine, and 4. Healthy colon mucosa has a different expression of factors, particularly miRNAs compared with colorectal cancer.

In inflammation such as that seen in allergy and respiratory inflammation, TSLP may be a driver of T<sub>H</sub>2 differentiation, proliferation and function and thus causal in these non-destructive inflammatory conditions. An alternative role for TSLP could be in its expression which is triggered as part of the inflammatory response as an attempt to maintain homeostasis and a non-inflammatory environment. In UC, a situation akin to more invasive infection and more destructive inflammation, pro-inflammatory factors come to the fore and drive a more aggressive inflammation with features leaning towards a T<sub>H</sub>1 response. In practice this is reflected by the beneficial effects of Infliximab, a TNF- $\alpha$  inhibitor, on more aggressive forms of UC.

The decreased expression of TSLP in mucosal biopsies reflects the combined expression from all cells within the mucosa including; epithelial cells, lymphocytes, monocytes and various other cells. A technique of analysing the origin of TSLP protein from within a sample is immunohistochemistry, however the study moved directly into cell subtype analysis and in vitro work partly because of logistical limitations of human sample collection and also the established association and interest in lymphocyte malfunction and UC, and the increased expression of miR-31 already shown in lymphocytes.

We have shown for the first time the expression of TSLP by mucosal lymphocytes in health and UC. Like previous studies we have shown that peripheral lymphocytes do not express TSLP. However we have also indicated that when stimulated there is a measurable expression of TSLP in 33% of lymphocytes at 24 hours and 66% at 48 hours, indicating that the expression of TSLP may be a

feature of activated lymphocytes. Lymphocytes extracted from the healthy sigmoid mucosa constitutionally express TSLP. This may be because a particular subset of lymphocytes is homed to the GI tract mucosa, or that exposure to the mucosal environment stimulates lymphocytes to express TSLP. The nature of this phenomenon remains to be found, but ECs are known to increase TSLP expression in response to bacteria, in much the same way as healthy mucosal lymphocytes increase TSLP expression when cultured with PHA.

Two initial studies had shown the expression of miR-31 and TSLP in PBMCs from healthy volunteers before and after stimulation with PHA, section 6.4.1 and 6.3.5 respectively. Further work on the expression and relationship between miR-31 and TSLP in lymphocytes however, involved using Jurkat cells as an *in vitro* cell model. This is because gene silencing, which is a key component in the investigation of the effect of altering miR-31 expression on TSLP involves transfection with an antagomir to miR-31. Primary lymphocytes are fragile and notoriously difficult to transfect, in contrast to Jurkat cells which show > 50% gene silencing in this study, Figure 51. There are a number of reasons why primary lymphocytes are challenging to transfect, including cell fragility and transfection toxicity which may explain why the manufacturer of INTERFERin™ only lists robust adherent cells in its list of successfully transfected primary cell lines (Polyplus Transfection Inc, 2012).

Jurkat cells are a well established *in vitro* cell model to analyse T lymphocyte function. The miR-31 and TSLP change seen with PHA stimulation in Jurkat cells mimics that seen in mucosal lymphocytes, Jurkat cells therefore provide a good model to infer the effect of miR-31 manipulation on TSLP in lymphocytes.

Jurkat cells cultured with PHA reveal a number of important findings; the first is the duration and concentration of PHA required for maximal stimulation. It is shown that PHA stimulation in terms of miR-31 expression reaches a plateau at 48 hours and shows a peak of expression at a concentration between 2.5 and 5.0 µg/ml. The activation of cells by PHA is situation specific, studies have used between 0.5 and >10 µg/ml to stimulate cells, and for durations up to several months. Higher doses cause increased cell death amongst certain cell types and

greater proliferation amongst others, longer durations are used to achieve cell proliferation rather than activation.

The expression of miR-31 increases and TSLP decreases in Jurkat cells stimulated with PHA. In individual cell cultures the association between the degree of miR-31 increase and the degree of TSLP decrease is inversely correlated,  $r_s = 0.905$ ,  $p = 0.002$ . This strong correlation implies that miR-31 plays a role in the TSLP pathway.

To study the effect of miR-31 “loss of function” on lymphocytes we introduced the antagomir of miR-31 into the previously described Jurkat cell model.

The use of antagomirs is an established method to study the effect of a “loss of function” of a particular gene (Kruzfeldt et al., 2005). To transfect the antagomir into the target cell it is first complexed with a generic siRNA transfection reagent (TRANSFERin™), this aids the introduction of the antagomir into the target cell during culture. Within the cell the antagomir binds with its complimentary base pair combination and prevents miRNA integration into the RISC complex and its function.

The efficiency of antagomir silencing is largely dependent on transfected cell type and technique. Under optimal conditions transfection with TRANSFERin™ can achieve over 90% efficiency (Polyplus Transfection Inc, 2012), however, Jurkat cell transfection is notoriously difficult with efficiencies ranging from <1% (Choi et al., 2012) to 70% (Jordan et al., 2008). There are no references to the efficiency of Jurkat cell transfection with INTERFERin™. Following the manufactures protocol we achieved a 50% reduction in the expression of miR-31 with no increase in cell death between transfected cells and cells grown in parallel culture.

The expression of TSLP mRNA and concentration of TSLP protein in cell culture supernatants are decreased in Jurkat cells stimulated with PHA and associated with an increased expression of miR-31. In cell cultures transfected with the antagomir to miR-31, there is no increase in miR-31 expression and no reduction in TSLP mRNA expression or production of TSLP, indicating a role for miR-31 in the TSLP pathway.

## Chapter 6. The association between miR-31 and TSLP

Interestingly the effect on TSLP protein production appears to be approximately twice the change seen for TSLP mRNA. In stimulated Jurkat cells transfected with a scrambled sequence antagomir the expression of TSLP mRNA is 0.71 fold compared with control, in the same cultures the concentration of TSLP protein is 0.46 fold compared with control, indicating a none linear relationship between TSLP mRNA expression and protein production.

The ratio of protein production to mRNA expression is dependent on many factors including mRNA degradation rate and ribosomal binding factors, the later is a feature of miRNAs. Despite the control of gene translation into protein by miRNAs, the production of protein could occur freely due to the phenomenon of “miRNA escape”. There are 3 variants of the TSLP protein gene, complementary binding of miR-31 to the 3'UTR of TSLP occurs in only one of the three variants, NCBI Reference Sequence: NM\_033035.4. The 3'UTR of this variant is 2570 bases in length compared with 57 and 719 for the other two. The translational repression of many protein coding genes occurs more frequently with those variants with longer 3'UTRs, probably due to increased base pair combinations. Deliberate shortening of the 3'UTR of a protein to escape miRNA inhibition is a feature of certain viral survival strategies (De Francesco and Migliaccio, 2005) and drug resistant cancer pathways (To et al., 2009). What degree of TSLP function depends on the three TSLP mRNA variants and therefore fall under the influence of miR-31 is unknown.

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## **Chapter 7. Discussion and future work**

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## 7.1 Conclusions

1. Seven miRNAs have a consistently deregulated expression in the sigmoid mucosa of patients with UC compared with healthy controls.
  - a. The expressions of miR-31, miR-146b and miR-223 have a consistently increased expression in the mucosa of active UC compared with healthy controls.
  - b. The expressions of miR-31 and miR-223 are also increased in the sigmoid mucosa of inactive UC compared with healthy controls.
  - c. The expressions of miR-194, miR-200b, miR-375 and miR-422a have a consistently decreased expression in the mucosa of active UC compared with healthy control.
  - d. The expression of miR-375 is also reduced in the sigmoid mucosa of inactive UC compared with healthy controls.
2. The expressions of miR-31 and miR-223 are increased, and miR-194 and miR-200b decreased in mucosal lymphocytes taken from active sigmoid UC compared with healthy control.
3. TSLP is identified as a target for 4 of the 7 miRNAs using in silico analysis.
4. The expression of TSLP mRNA is 39% less in the mucosa of active UC compared with healthy controls.
5. The expression of TSLP protein in active UC is 34% of the expression in healthy controls.
6. TSLP is not expressed in unstimulated PBMCs, but is expressed when PBMCs are stimulated with PHA.
7. There is a constitutional expression of TSLP in mucosal lymphocytes.
8. The expression of TSLP mRNA is 61% less in mucosal lymphocytes and 33% less in CD4<sup>+</sup>CD25<sup>intermediate</sup> mucosal lymphocytes of active UC compared with healthy control.
9. The expression of miR-31 and TSLP mRNA is inversely correlated in stimulated Jurkat cells.
10. Silencing of miR-31 abrogates the effect of PHA stimulation on TSLP in Jurkat cells indicating a role for miR-31 in the TSLP pathway.

## 7.2 Discussion

### 7.2.1 Animal models

The initial hypothesis of this thesis is that in IBD there is a distinct number of miRNAs that are differentially expressed compared with healthy individuals and that one or all of these miRNAs influence an inflammatory pathway in such a way as to predispose to or perpetuate IBD. Murine models are a well established method of reproducing inflammatory conditions akin to that seen in IBD. We started by attempting to develop a murine model which would mimic the features of IBD in terms of miRNA and cytokine expression.

The initial experiments analysed the expressions of miR-155 and miR-125 in 3 widely used models of chemically induced experimental colitis; TNBS, Oxazolone and DSS. It was expected that in induced colitis the expression of miR-155 would be significantly increased with an inversely related expression of miR-125. At the time there was no direct evidence of raised miR-155 in IBD in fact the only miRNA profile in human mucosa at the time had not demonstrated an increase in miR-155 (Wu et al., 2008), however the expected increase was based on the well established role of miR-155 in many types of in vitro and animal models of inflammation. The inverse relationship between miR-155 and miR-125 had been demonstrated in LPS stimulated murine monocytes and thought to be a result of TNF- $\alpha$  driven miRNA expression (Tili et al., 2007). TNF- $\alpha$  continues to be the prominent pro-inflammatory cytokine and target for biological therapy in IBD it therefore stands to reason that a similar relationship should be seen in humans.

The increased expression of miR-155 in IBD has since been confirmed (Takagi et al., 2010, Van der Goten et al., 2012). The expression of miR-125 does not feature as a differentially expressed miRNA in any of the microarray studies to date, although tissue used for these are mucosal biopsies, which may indicate that miR-125 has a cell specific expression e.g. down regulated in healthy T<sub>regs</sub> compared with other T cells (Rouas et al., 2009), and decreased in stimulated monocytes, therefore not applicable to whole biopsy studies.

In all three experimental models we demonstrated an increased expression of miR-155 compared with healthy controls, although the results were not consistent and only seen in certain variants of each model; including 3.0% TNBS, but not 2.0% or 2.5% TNBS, after day 3 but not day 5 of the 2% Oxazolone model, and with the 2% DSS model. The expression of miR-125 appeared to follow no pattern across the models, with a small but significant increase in both the 2.5% and 3.0% TNBS but not the 2.0% model, and no significant difference in the DSS model or in either the day 3 or day 5 variant of the Oxazolone model.

The increase in miR-155 was greatest in the day 3 variant of the 2% Oxazolone model, and therefore cytokines were analysed in this model.

The premise of experimental models is that the cytokine expression profile mimics that of either UC or Crohn's, i.e. in Crohn's like colitis the expression of IL-12, IL-23, IFN $\gamma$  and TNF- $\alpha$  are increased and in UC the expression of IL-4, IL-5 and IL-13 are increased.

The results presented in Chapter 3 show that 2% Oxazolone did not favour a polarised T cell response. On day 3 there was an increase in TNF- $\alpha$  but not INF- $\gamma$ , and an increased expression of IL-4, IL-17 and IL-10. On day 5 the expression of FOXP3 was increased suggesting an increase activity of regulatory T cells with no change in the expression of TNF- $\alpha$ , IL-4, IL-17 or IL-10, although TGF- $\beta$  continued to increase.

Taken together these results demonstrate that under the experimental conditions used, the 2% Oxazolone model had a cytokine profile consistent with a generalised mucosal inflammation which did not mimic either a Crohn's like (T<sub>H</sub>1) or UC like (T<sub>H</sub>2) type reaction and suggests that miR-155 is a generalised marker of inflammation rather than specific to a particular disease model.

Although attempts are made to categorise different models into either a T<sub>H</sub>1 or T<sub>H</sub>2 type inflammation, see review by Strober et al. (2002, p 498), the actual immune response as defined by cytokine pattern, histology or macroscopic pattern of disease depends on many factors including the mouse strain and experiment conditions. For example with TNBS models of colitis, in SJL/J mice a chronic T<sub>H</sub>1

## Chapter 7. Discussion and future work

mediated response is seen, but in  $\text{INF-}\gamma^{-/-}$  Balb/c mice a  $T_H2$  mediated colonic patch hypertrophy is seen. Oxazolone is often used to study  $T_H2$  pathways but at lower doses a mixed  $T_H1/T_H2$  dependent colitis is seen.

Dextran Sulphate Sodium, TNBS and Oxazolone are all widely used to study inflammation within the bowel and often interpreted as mimicking IBD, however they only resemble some aspects of human IBD (Wirtz and Neurath, 2007) and it is therefore impossible to unambiguously classify the different mouse models into UC or Crohn's type inflammation (Celinski et al., 2010).

In an attempt to further understand the miRNA profile and cytokine targets in human IBD, treatment naive, active sigmoid UC biopsies were used, focusing on active UC.

### 7.2.2 The miRNA profile of the sigmoid mucosa, mucosal lymphocytes and $\text{CD4}^+\text{CD25}^{\text{intermediate}}$ mucosal lymphocytes

To date there are a number of published (Wu et al., 2008, Fasseu et al., 2010, Takagi et al., 2010) and unpublished (Van der Goten et al., 2012) mucosal miRNA profiles for UC. Together these have identified 65 miRNAs that have a differential expression between active UC and healthy controls. The functional relevance of these miRNAs has yet to be demonstrated in humans, although in vitro and animal models have suggested multiple pathways through which many of these miRNAs could play a role.

This study has identified 7 miRNAs that have a consistent differential expression between biopsies taken from active sigmoid UC and healthy controls. Although all 7 have been previously identified by individual microarrays the concordance between studies has been poor, mainly because of differing patient phenotype and microarray methodology. It is also established that cell, tissue and regional differences in miRNA expression make comparison across these studies difficult (Wu et al., 2010).

Sample heterogeneity challenges the interpretation between studies and also limits their clinical relevance. Existing studies have combined samples collected

from various sources including; resections and biopsies, various regions of the colon, differing definitions of inactive disease and importantly reported miRNA expression in patients on a variety of immune altering treatments, including 5-ASAs, immunosuppressive medication and biologics. Heterogeneity makes interpretation of the clinical relevance of miRNAs challenging other than the broad theme that potentially large numbers of miRNA are deregulated in IBD.

The relevance of differentially expressed miRNAs in disease is their ability to explain disease aetiology and provide possible therapeutic targets. A real strength of this work is the homogeneity of the samples used. This study therefore takes into account several important variables in sample selection; human versus animal, biopsies taken at endoscopy rather than resection samples, colonic mucosa rather than blood, sigmoid rather than other colonic regions and samples from patients who are not on treatment that could potentially alter disease and / or miRNA expression.

This thesis starts by demonstrating a differential expression of 7 miRNAs in active UC compared with healthy controls. By comparing microarrays, first by ranking miRNA expression, then by only analysing those with consistently deregulated expression, all 7 miRNAs are validated by RT-qPCR. Previous studies reporting comparative expressions analysed by microarray alone give a false positive rate, and can increase the number of differentially expressed miRNA by up to 200%, these studies should be interpreted with caution.

The expressions of the 7 candidate miRNAs are reported in 3 distinct areas; the first is in mucosal biopsies, the second in mucosal lymphocytes derived by centrifugation on a Percoll gradient and the second is in CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocytes derived initially on a Percoll gradient and then acquired with flow cytometry.

The miRNA expressions in the mucosa are the most widely reported profiles in mucosal UC. This is because of the logistics of tissue collection and quantity. This however provides an expression profile for a mixed cell population, with different and unknown proportions of each cell type within samples, for example there is likely to be a higher proportion of epithelial cells in biopsies of the same size from

## Chapter 7. Discussion and future work

normal mucosa compared with inflamed mucosa, and alternatively a higher proportion of inflammatory immune cells in actively inflamed mucosa compared with healthy mucosa. The cellular origin of the miRNA is therefore impossible to conclude from whole mucosal biopsies; however what can be concluded is the differential miRNA environment between active UC and health.

Density centrifugation of digested biopsy tissue achieved a cell suspension of 32.2% lymphocytes with  $\geq 90\%$  viability. This again is a mixed population including lymphocytes, but is considerably more enriched with lymphocytes than whole biopsies. There are no reported miRNA expressions in mucosal lymphocytes extracted from biopsies. In this population the expression of 4 of the 7 miRNAs continue to have a significant differential expression compared with healthy lymphocytes. The expressions of miR-31 and miR-223 are higher, and miR-194 and miR-200b are lower in active UC.

Using flow cytometry and multicolour analysis of the lymphocyte cell solution acquired through density centrifugation, a population of CD3<sup>+</sup>CD4<sup>+</sup> lymphocytes was initially acquired, and then further divided depending on their CD25 characteristics. CD25 is a cell surface antibody expressed on the surface of activated lymphocytes; these include both pro-inflammatory and regulatory T cells. In the periphery it has already been demonstrated that T<sub>regs</sub> express a different miRNA profile to other CD4<sup>+</sup> cells, in part due to the influence of miRNAs in controlling cellular function, particularly the negative influence of miR-31 on FoxP3 expression (Rouas et al., 2009, Hezova et al., 2009), it is therefore important to separate these functionally opposite cell populations.

Regulatory T cells are characterised by the expression of FoxP3, an intercellular transcription factor. To identify FoxP3 within cells requires fixation and permeabilization, a process which traditionally results in RNA cross linking and degradation. In some techniques, such as formalin fixed paraffin embedded samples, small RNA escape the effects of permeabilization and can therefore be reliably measured (Li et al., 2007, Xi et al., 2007), however for flow cytometry newer techniques which offer a way of measuring certain gene expressions after intracellular antigen staining (Yamada et al., 2010), have not yet been applied to

the measurement of miRNAs. To date the conventional method of separating  $T_{regs}$  from a heterogeneous T cell pool is by surface antigen characteristics.

Regulatory T cells express high levels of surface CD25. In peripheral blood 80 - 95% of  $CD4^+CD25^{high}$  cells stain for FoxP3 although the proportion is slightly less in IBD (Roncador et al., 2005, Eastaff-Leung et al., 2009), indicating that in health a larger proportion of activated peripheral  $CD4^+$  cells are of the regulatory phenotype. In the colonic mucosa it is accepted that the number of lymphocytes increases with the severity of IBD and that the number of  $CD4^+CD25^{high}$  are also increased (Holmen et al., 2006), although the proportion of  $T_{regs}$  is less in IBD compared with non IBD colitis (Maul et al., 2005). The proportion of  $CD4^+CD25^{high}$  that also stain for FoxP3 in the colonic mucosa is not known although FOXP3 mRNA expression is 2 - 5 times higher in colonic  $CD4^+CD25^{high}$  compared with  $CD4^+CD25^{low}$ , and  $CD4^+CD25^{high}$  but not  $CD4^+CD25^{low}$  exert a suppressive effect on T cell proliferation and cytokine production (Makita et al., 2004). All this together suggests that like blood, most sigmoid  $T_{regs}$  are within the  $CD4^+CD25^{high}$  population.

Exclusion of 20% of the most brightly stained CD25 lymphocytes ( $T_{regs}$ ) and 10% of the least brightly stained lymphocytes (naive T cells), leaves the  $CD4^+CD25^{intermediate}$  cell sub family. This was shown to be only 4% FoxP3<sup>+</sup>, and therefore  $T_{regs}$ , an important finding because the influence of  $T_{regs}$  miRNA expression on the group would be negligible. The expression of miR-31 and miR-223 was again significantly increased in  $CD4^+CD25^{intermediate}$  from active UC compared with healthy controls.

It is clear that certain miRNA have a purposeful influence on biochemical pathways beyond coincidental interaction and those influences are finely balanced, probably with many other factors, to control expression and function of many protein coding genes. Dysfunction of miRNA regulation within distinct pathways, particularly involving inflammation, is likely to influence disease predisposition, initiation and propagation. The expression of miRNAs in different cell types is likely to reflect the need to control the expression of different genes under differing circumstances.

TSLP was identified as a potential target of 4 of the 7 miRNAs by bioinformatic algorithms, and importantly it is a predicted target for miR-31, a miRNA which has

shown a consistent increase in expression in active UC compared with healthy control. We also show that in association with an increased miR-31 the expression of TSLP is reduced in the mucosa, mucosal lymphocytes and mucosal CD4<sup>+</sup>CD25<sup>intermediate</sup> lymphocytes of active UC.

### 7.2.3 TSLP in IBD and regulation by miR-31

It is almost 20 years since the biological function of TSLP was first described (Friend et al., 1994) and although well documented in disorders of the skin and lung it was not until 2000 that murine studies identified TSLP in the ileum (Sims et al., 2000). It has since been shown that TSLP is constitutionally expressed by the human colonic mucosa (Rimoldi et al., 2005).

Much of what we know about human TSLP comes from work on T<sub>H</sub>2 mediated inflammatory disorders of the skin and lung in which the expression of TSLP is increased. Murine studies also contribute towards our knowledge of TSLP but must be interpreted with caution because of the inter-species differences in the origin, expression and action of TSLP. In the human TSLP is thought to originate only from epithelial cells and keratinocytes at barrier surfaces in response to antigens or bacterial invasion, in mice TSLP is expressed by other cells, predominantly dendritic cells, independently of epithelial cells. The action of Human TSLP is through the STAT5 pathway, in mice TSLP works also through the STAT3 pathway. Differences may be due to poor sequence homology between humans and mice, only 43%, which could also result in other subtle, as yet unknown, differences of function.

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- $\alpha$  and IL-4 (Tanaka et al., 2010), reviewed by Takai (2012).

In response to commensal flora TSLP aids in the maintenance of mucosal homeostasis, by maintaining immune hypo-responsiveness through the promotion

of  $T_H2$  cell maturation and proliferation. When challenged by certain pathogens particularly Helminths, TSLP is critical in developing the defensive  $T_H2$  mediated cytokine response and pathogen expulsion. Abnormal TSLP expression is also found in other infections such as H Pylori in human gastric cell lines (Kido et al., 2010) and Rhinovirus in human bronchus epithelial cells (Kato et al., 2007) In the GI tract there are regional differences in TSLP expression which may be explained by the regional differences in the microbiome (Taylor et al., 2009).

TSLP is multifunctional cytokine with an important role in Dendritic cell activation, T cell maturation and  $T_H2$  cell proliferation (Reche et al., 2001, Soumelis and Liu, 2004).

In health epithelial cells constitutionally express TSLP but little is known about TSLP in IBD. It is commonly suggested that TSLP is not expressed in the colon of active Crohn's (Biton et al., 2011) although this is not a complete statement and misleading. In the original study by Rimoldi et al. (2005), the analysis of TSLP by qPCR is from unaffected mucosa (region not described) of patients undergoing resection for Crohn's (phenotype not described). There is undetectable expression of TSLP mRNA in 6 of the 9 Crohn's samples, and of note is the reduced TSLP mRNA expression in 2 out of the 3 controls. No other study confirms or refutes this finding.

It is suggested that the increased expression of TSLP in  $T_H2$  disorders indicates a "*cycles of amplification*" (Takai, 2012), in which TSLP expression is triggered and inappropriately exaggerated leading to the inflammatory response seen. Ulcerative colitis has traditional thought of as a  $T_H2$  mediated inflammatory condition, and it stands to reason that TSLP should be increased in the mucosa. It has been reported that TSLP expression is raised in UC, although in the original study Tanaka et al. (2010) compared surgical resections of UC against patients undergoing colorectal cancer treatment, this is a unique UC phenotype who are probably on steroids compared with colorectal cancer patients who are not "healthy controls" and potentially have a differential expression of miRNAs of their own.

## Chapter 7. Discussion and future work

The findings of Tanaka et al. (2010) fit with the theory of raised TSLP in  $T_H2$  mediated inflammation. However, we have shown that TSLP has a decreased expression in UC. The explanation for this dichotomy may be due to a number of factors including; the differences in disease aetiology between allergic lung inflammation and UC which is a disordered and often destructive inflammatory reaction with features of a  $T_H1$  mediated response. The opposing results of Tanaka et al. May be explained by differences in sample population, disease phenotype and treatment regimes, which are known to alter the factors which influence TSLP expression. It is interesting also that cigarette smoking, a therapy that can protect against UC, can induce TSLP production in ECs (Nakamura et al., 2008) something that would only help if a decreased TSLP expression was part of the aetiological milieu of UC.

Human studies have been unable to identify TSLP expression from peripheral or GI tract lymphocytes. The mechanism of action of TSLP on lymphocytes is through the influence of TSLP on DCs which then drive  $T_H2$  lymphocyte responses, although recently the TSLP receptor has been shown to be expressed by peripheral lymphocytes activated in vitro, whilst not expressed by naive peripheral  $CD4^+$  lymphocytes. In-vitro activation of naive peripheral lymphocytes with anti-CD3<sup>+</sup> and co-culture with TSLP, results in increased proliferation and increased sensitivity of lymphocytes to CD2 (Rochman et al., 2007), suggesting that like mice, TSLP can act directly on lymphocytes and increase their inflammatory response.

The connection between miRNA and TSLP has not been shown in IBD beyond the predicted targeting in 4 of the 7 miRNAs. In  $Dicer^{A_{gut}}$  mice which have reduced levels of miRNA, a reduced expression of TSLP is found (Biton et al., 2011) suggesting miRNA are necessary for TSLP expression. We show that in the three aspects of active UC; mucosa, mucosal lymphocytes and  $CD4^+CD25^{intermediate}$  mucosal lymphocytes the expression of miR-31 is increased and TSLP is decreased. Using an in vitro lymphocyte model we have demonstrated an inverse relationship between miR-31 and TSLP, and confirmed the ex vivo finding in extracted lymphocytes that stimulation increases miR-31 and decreases TSLP expression.

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.

Although CD4<sup>+</sup> T cells have been shown to express the TSLP receptor, this is the first study to identify TSLP expression in lymphocytes. It is possible that both TSLP and TSLP receptor expression form a feedback loop to maintain immune homeostasis through prevention of an over aggressive immune response.

The complexity of the immunological milieu and heterogeneity of disease phenotypes has confounded attempts to discover the aetiology of IBD. We have shown that the expression of certain miRNAs in UC is increased and certain miRNAs are decreased. We have demonstrated that miR-31 is associated with a decreased level of TSLP and that manipulating miR-31 expression directly effects TSLP expression.

Both miR-31 and miR-223 have an increased expression in the sigmoid colon of patients who have no macroscopic inflammation at the time of endoscopy indicating that these miRNAs have a greater expression in the mucosa of patients with UC than healthy controls even in the absence of inflammatory activity. These miRNAs are potential diagnostic markers for UC, suggesting they could potentially be used as a marker for diagnosis in the absence of macroscopic disease.

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. On one hand UC tends towards a T<sub>H</sub>2 inflammatory pattern but on the other there are strong features of T<sub>H</sub>1 pattern as well as many others. 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, the breakdown of regulation of the epithelial barrier, and the predominance of T<sub>H</sub>1 type responses in more severe UC phenotypes. 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.

### 7.3 Future work

Despite the growing evidence that miRNAs have a crucial influence on human disease our understanding of these processes are in their infancy. What the total number of miRNAs will reach is unknown, although it is clear that many of them play a pivotal role individually, or in combination, in many distinct and / or generalised inflammatory pathways. In this study we have focused on the influence of miR-31 on TSLP in UC.

The use of miRNAs as a novel screening tool in IBD as well as other illnesses is receiving growing attention (Iborra et al., 2012). In chapter 4 it is shown that the expression of miR-31 is increased in mucosa biopsies of inactive and active UC compared with health. There are also 6 miRNAs that are identified as consistently deregulated in active UC. Alone or in combination with other miRNAs, miR-31 has promise as a useful diagnostic or screening tool, however miR-31 and the expressions of other miRNAs are not UC specific (Sonkoly and Pivarcsi, 2009), requiring further studies to compare miRNA profiles of UC with other forms of colitis and the relationship between mucosal and peripheral expression.

In chapter 5, it has been shown that miR-31 has an increased expression in CD4<sup>+</sup>CD25<sup>intermediate</sup> mucosal lymphocytes. MiR-31 is expressed from a wide variety of cell types (Valastyan and Weinberg, 2010) and it stands to reason that other cell types contained within the mucosal biopsies such as epithelial cells, monocytes or other lymphocyte subsets are also likely to contribute to the miR-31 expression in the mucosa. This thesis did not extract other cell subsets from the mucosa, partly due to logistical issues, but also because the focus was centred on lymphocytes. To understand the functional significance of miR-31 and effect on other prominent factors associated in the aetiology of IBD understanding the spatial expression pattern of miR-31 within the colonic mucosal would be helpful.

In chapter 6 it is shown that there is a decreased expression of TSLP in the mucosa, mucosal lymphocytes and mucosal CD4<sup>+</sup>CD25<sup>intermediate</sup> cell population. To date, studies suggest that TSLP expression drives certain disease conditions. However, in these situations a raised TSLP may be physiologically normal, part of the bodies attempt to steer inflammation away from more destructive type of

inflammation and thus regulate immunity. A decreased TSLP expression may therefore indicate an inability to regulate inflammation and thus cause or potentiate disease. Whether or not TSLP drives inflammation or is part of a regulatory process, the expression of TSLP and its function may well be influenced by many other yet unknown factors such as time from insult, degree of insult and the therapeutic range of expression and influence.

It has already been shown that activated peripheral lymphocytes express the TSLP receptor (Rochman et al., 2007), but the expression of TSLP directly has not previously been shown. The expression of TSLP by an increasing number of cell types would suggest that it has a functional significance beyond the traditional role of an epithelial derived factor that influences lymphocytes through a dendritic cell pathway. Autocrine signalling is a potential positive feedback mechanism through which TSLP regulates an inflammatory response in activated lymphocytes, the lack of expression of both TSLP receptor and TSLP in naive lymphocytes may allow these naive lymphocytes to respond more rapidly to external factors, before the regulatory mechanism of TSLP is engaged.

The functional significance of TSLP in lymphocytes has yet to be explored and is not addressed in this thesis. The function of TSLP is traditionally thought to be through STAT5 signalling (Rochman et al., 2007), however Takai (2012) and Taylor (2009) review multiple mechanisms which are potentially involved in the expression and function of TSLP and suggests there are many other factors which are yet unknown, possibly cell specific and yet to be explored.

Questions raised by this study include;

1. If Jurkat cells and primary lymphocytes express TSLP in response to activation do they also express the TSLP receptor and provide the hypothesized positive feedback mechanism?
2. Through what mechanism does TSLP exert its effect? Does lymphocyte / Jurkat cell stimulation activate the STAT5 pathway or an alternate pathway?
3. What is the functional significance of TSLP?
  - i. Does TSLP alter the lymphocyte phenotype?

## Chapter 7. Discussion and future work

- ii. Does TSLP play a role in lymphocyte proliferation, maturation and migration?
4. Does TSLP play a differing role in different types or degrees of inflammation?

It is yet to be revealed if miR-31 or TSLP alone are sufficient to influence mucosal immune responses. We have shown that miR-31 provides a mechanism through which TSLP mRNA can be manipulated; however there are many other factors which influence its expression and function and need to be explored. We have identified constitutional expression of TSLP by mucosal lymphocytes, along with TSLP receptor expression in activated lymphocytes this offers a potential insight into an as yet unexplored positive feedback mechanism.

Like so many other potentially exciting therapies for UC, the efficacy of therapy between individuals differs for unexplained reasons and declines over time. The relevance of protein variants and “miRNA escape” mechanisms are becoming increasingly recognised in viral pathways, and could be relevant in UC and explain differences in disease patterns between otherwise identical patients.

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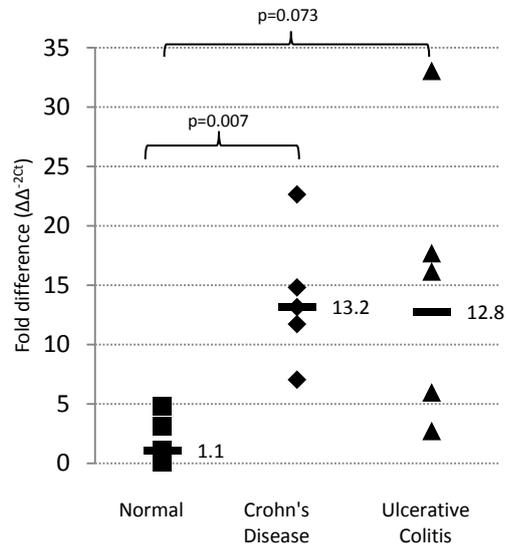
## **Chapter 8. Appendix**

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## 8.1 Preliminary data

### 8.1.1 miR-155 expression in IBD (unpublished results)



**Figure 53.** The differential expression on miR-155 in IBD, preliminary results. The expression of miR-155 in mucosal resection specimens from patients with Crohn's (n = 5) and UC (n = 5) are compared against the expression of miR-155 in the mucosa of patients undergoing colectomy for colorectal cancer (n = 5) using RT-qPCR. Results reproduced courtesy of Dr Rebecca Morgan-Walsh. Mean values of each group are compared with the two tailed student's t test.

### 8.1.2 miR-155 expression in a 3% TNBS model of colitis

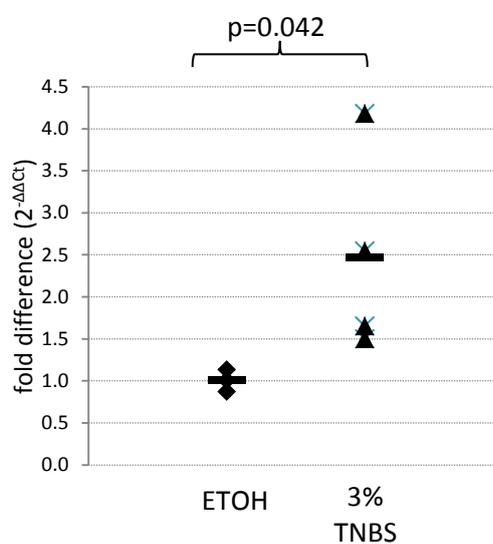


Figure 54. The differential expression on miR-155 in a 3% TNBS model of colitis, preliminary results. The expression of miR-155 in the mucosa of mice is compared 4 days after rectal infusion of either 3% TNBS (n = 4), or control/ETOH (n = 4) using RT-qPCR. Experiments performed by Dr Elena Vorobeva. Mean values of each group are compared with the two tailed students t test.

## 8.2 Patient data collection sheet

Version 5 (updated 1<sup>st</sup> Sept 2011)

Date: \_\_/\_\_/\_\_

Biopsy N<sup>o</sup>: \_\_\_\_\_**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<sup>1</sup>
10. Maximal extent of inflammation \_\_\_\_\_  
Date \_\_\_\_\_
11. Normal day N<sup>o</sup> of stools \_\_\_\_\_  
N<sup>o</sup> of loose stools \_\_\_\_\_
12. Last day N<sup>o</sup> of stools \_\_\_\_\_  
N<sup>o</sup> of loose stools \_\_\_\_\_
13. Last week N<sup>o</sup> of stools \_\_\_\_\_  
N<sup>o</sup> of loose stools \_\_\_\_\_
14. Bleeding<sup>2</sup> (in last day)  
N<sup>o</sup> of stools with blood \_\_\_\_\_  
 no  
 blood < 1/2 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<sup>3</sup>  
 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<sup>4</sup>**
- i. = \_\_\_\_\_
- ii. = \_\_\_\_\_
- iii. = \_\_\_\_\_

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

<sup>3</sup> 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.

<sup>4</sup> 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

## 8.3 Microarray data

## 8.3.1 Expression of miRNA in active UC

	mean	±sd	t test		mean	±sd	t test
miR-31	35.257	0.782	<0.001	miR-106b	0.752	0.346	0.282
miR-223	8.523	2.424	0.047	miR-487a	0.748	0.389	0.300
miR-486-3p	4.588	1.440	0.076	miR-125a-5p	0.747	0.104	0.137
miR-708	3.960	0.586	0.062	miR-196b	0.745	0.187	0.232
miR-146b-5p	3.766	1.223	0.076	miR-320	0.737	0.012	0.010
miR-483-5p	3.742	0.490	0.018	miR-195	0.727	0.537	0.330
miR-628-5p	3.292	1.587	0.152	miR-411	0.725	0.079	0.238
miR-132	2.985	2.164	0.230	miR-452	0.712	0.006	0.192
miR-142-3p	2.809	1.675	0.198	miR-18a	0.702	0.258	0.262
miR-155	2.715	1.310	0.166	miR-106a	0.699	0.371	0.251
miR-18b	2.562	1.849	0.252	let-7e	0.699	0.558	0.321
miR-150	2.500	0.805	0.106	miR-199a-3p	0.688	0.403	0.260
miR-135b	2.489	1.927	0.284	miR-374b	0.667	0.356	0.224
miR-539	2.488	1.986	0.358	miR-95	0.653	0.235	0.139
miR-146a	2.471	1.033	0.146	miR-191	0.643	0.112	0.076
miR-126	2.350	1.046	0.163	miR-20b	0.643	0.452	0.261
miR-331-5p	2.154	0.656	0.233	miR-139-5p	0.633	0.320	0.205
miR-21	2.085	1.233	0.253	miR-93	0.632	0.356	0.216
miR-501-5p	2.044	1.101	0.223	miR-29c	0.620	0.258	0.206
miR-125b	2.040	1.376	0.266	miR-660	0.614	0.454	0.242
miR-193b	1.921	0.236	0.040	miR-133b	0.609	0.521	0.270
miR-148b	1.881	1.038	0.243	miR-210	0.607	0.007	0.228
miR-494	1.714	1.431	0.377	miR-574-3p	0.595	0.136	0.054
let-7f	1.674	1.349	0.343	miR-26b	0.594	0.415	0.216
miR-140-3p	1.633	0.494	0.164	miR-886-5p	0.592	0.044	0.156
let-7c	1.628	1.190	0.337	miR-186	0.578	0.016	0.001
miR-99a	1.590	1.461	0.380	miR-193a-5p	0.576	0.550	0.262
miR-202	1.555	0.776	0.405	miR-337-5p	0.564	0.142	0.243
miR-454	1.525	0.761	0.379	miR-19b	0.563	0.404	0.197
miR-23b	1.523	1.288	0.406	miR-20a	0.543	0.396	0.184
miR-15a	1.489	0.029	0.433	miR-133a	0.523	0.259	0.104
miR-134	1.412	0.447	0.328	miR-103	0.517	0.370	0.162
miR-34a	1.361	0.775	0.381	miR-149	0.514	0.365	0.195
miR-212	1.343	0.086	0.033	miR-491-5p	0.508	0.055	0.044
miR-652	1.343	0.955	0.409	miR-127-3p	0.506	0.386	0.214
miR-222	1.341	0.599	0.314	miR-218	0.505	0.311	0.188
miR-224	1.338	0.954	0.379	miR-370	0.504	0.060	0.034
miR-455-5p	1.337	0.278	0.252	miR-130b	0.503	0.029	0.028
miR-365	1.326	1.191	0.405	miR-28-3p	0.502	0.297	0.118
miR-140-5p	1.289	0.824	0.384	miR-10a	0.502	0.279	0.139
miR-342-3p	1.261	0.388	0.312	miR-345	0.493	0.134	0.034
miR-214	1.230	0.067	0.351	miR-339-3p	0.482	0.155	0.040
miR-181a	1.226	0.738	0.398	miR-26a	0.476	0.340	0.133
miR-16	1.225	0.109	0.129	miR-425	0.474	0.128	0.030
let-7a	1.174	0.931	0.445	miR-532-3p	0.461	0.182	0.096
miR-100	1.148	0.909	0.451	miR-203	0.460	0.182	0.066
miR-138	1.117	0.283	0.370	miR-92a	0.450	0.262	0.110
let-7b	1.097	0.699	0.457	miR-145	0.440	0.378	0.157
miR-29b	1.094	0.227	0.493	miR-324-3p	0.423	0.121	0.033
miR-25	1.094	0.948	0.465	miR-486-5p	0.423	0.184	0.050
miR-99b	1.088	0.877	0.485	miR-101	0.418	0.334	0.121
miR-28-5p	1.062	0.489	0.460	miR-376c	0.416	0.142	0.027
miR-15b	1.051	0.922	0.499	miR-10b	0.415	0.379	0.194
miR-29a	1.038	0.563	0.485	miR-30b	0.413	0.327	0.110
miR-744	1.031	0.560	0.484	miR-328	0.411	0.336	0.135
miR-27a	1.025	0.631	0.486	miR-886-3p	0.406	0.189	0.050
miR-484	1.018	0.269	0.485	miR-27b	0.400	0.320	0.108
miR-331-3p	1.015	0.320	0.495	miR-30c	0.399	0.249	0.072
miR-24	1.000	0.404	0.490	miR-1	0.383	0.018	<0.001
let-7d	0.989	0.741	0.490	miR-197	0.381	0.247	0.076
miR-130a	0.973	0.582	0.451	miR-200b	0.331	0.080	0.031
miR-362-5p	0.948	0.729	0.475	miR-340	0.327	0.200	0.103
miR-221	0.939	0.518	0.454	miR-215	0.323	0.077	0.125
miR-148a	0.916	0.660	0.455	miR-532-5p	0.322	0.159	0.042
miR-185	0.875	0.336	0.374	miR-200a	0.309	0.059	0.004
miR-451	0.865	0.757	0.423	miR-379	0.283	0.092	0.024
miR-339-5p	0.845	0.769	0.425	miR-200b	0.251	0.124	0.014
miR-590-5p	0.817	0.344	0.327	miR-194	0.213	0.058	0.020
miR-152	0.812	0.013	0.132	miR-141	0.212	0.042	0.002
miR-518d-3p	0.807	0.434	0.316	miR-192	0.199	0.056	0.004
miR-374a	0.782	0.461	0.342	miR-429	0.178	0.077	0.011
miR-19a	0.778	0.541	0.361	miR-375	0.129	0.045	0.002
miR-376a	0.767	0.367	0.313	miR-422a	0.106	0.005	<0.001
miR-338-3p	0.758	0.464	0.328				
miR-17	0.754	0.447	0.319				
miR-143	0.753	0.565	0.345				
let-7g	0.752	0.472	0.326				

Table 45. miRNAs differentially expressed in active sigmoid UC. Microarray was performed using TaqMan® Array Human MiRNA A Cards v2.0. 150 miRNA had an expression below the threshold CT ≤ 35 in all array card pairs. miRNA expression was normalised to MammU6 and compared against healthy control. Microarray data is

presented as mean fold difference of active UC and analysed using the one tailed unpaired students t test.

### 8.3.2 Non expressed miRNA in active sigmoid UC

miRNA					
miR-105	miR-219-5p	miR-372	miR-501-3p	miR-520d-5p	miR-615-5p
miR-107	miR-219-a	miR-373	miR-502-3p	miR-520e	miR-616
miR-122	miR-219-b	miR-376b	miR-502-5p	miR-520f	miR-618
miR-124	miR-22	miR-377	miR-503	miR-520g	miR-624
miR-125a-3p	miR-220	miR-380	miR-504	miR-521	miR-625
miR-127-5p	miR-220b	miR-381	miR-505	miR-522	miR-627
miR-128	miR-220c	miR-382	miR-506	miR-523	miR-629
miR-129-3p	miR-23a	miR-383	miR-507	miR-524-5p	miR-636
miR-129-5p	miR-296-3p	miR-384	miR-508-3p	miR-525-3p	miR-642
miR-135a	miR-296-5p	miR-409-5p	miR-508-5p	miR-525-5p	miR-651
miR-136	miR-298	miR-410	miR-509-3p	miR-526b	miR-653
miR-137	miR-299-3p	miR-412	miR-509-5p	miR-541	miR-654-3p
miR-139-3p	miR-299-5p	miR-423-5p	miR-510	miR-542-3p	miR-654-5p
miR-142-5p	miR-301a	miR-424	miR-511	miR-542-5p	miR-655
miR-146b-3p	miR-301b	miR-431	miR-512-3p	miR-544	miR-671-3p
miR-147	miR-302a	miR-433	miR-512-5p	miR-545	miR-672
miR-147b	miR-302b	miR-448	miR-513-5p	miR-548a-3p	miR-674
miR-153	miR-302c	miR-449a	miR-515-3p	miR-548a-5p	miR-758
miR-154	miR-32	miR-449b	miR-515-5p	miR-548b-3p	miR-871
miR-181c	miR-323-3p	miR-450a	miR-516a-5p	miR-548b-5p	miR-872
miR-182	miR-324-5p	miR-450b-3p	miR-516b	miR-548c-3p	miR-873
miR-183	miR-325	miR-450b-5p	miR-517a	miR-548c-5p	miR-874
miR-184	miR-326	miR-453	miR-517b	miR-548d-3p	miR-875-3p
miR-187	miR-329	miR-455-3p	miR-517c	miR-548d-5p	miR-876-3p
miR-188-3p	miR-330-3p	miR-485-3p	miR-518a-3p	miR-551b	miR-876-5p
miR-190	miR-330-5p	miR-485-5p	miR-518a-5p	miR-556-3p	miR-885-3p
miR-193a-3p	miR-335	miR-487b	miR-518b	miR-556-5p	miR-885-5p
miR-198	miR-33b	miR-488	miR-518c	miR-561	miR-887
miR-199a-5p	miR-342-5p	miR-489	miR-518d-5p	miR-570	miR-888
miR-199b-5p	miR-346	miR-490-3p	miR-518e	miR-576-3p	miR-889
miR-204	miR-34c-5p	miR-491-3p	miR-518f	miR-576-5p	miR-890
miR-205	miR-361-5p	miR-492	miR-519a	miR-579	miR-891a
miR-208	miR-362-3p	miR-493	miR-519c-3p	miR-582-3p	miR-891b
miR-208b	miR-363	miR-495	miR-519d	miR-582-5p	miR-892a
miR-211	miR-367	miR-496	miR-519e	miR-589	miR-9
miR-216a	miR-369-3p	miR-499-3p	miR-520a-3p	miR-597	miR-96
miR-216b	miR-369-5p	miR-499-5p	miR-520a-5p	miR-598	miR-98
miR-217	miR-371-3p	miR-500	miR-520b	miR-615-3p	

Table 46. miRNA that are not expressed in active sigmoid UC. Microarray data of none expressed miRNA including those miRNA which are amplified above the threshold  $C_T \geq 35$ . 217 miRNAs are not expressed in the microarray, a further 10 are expressed in  $\leq 2$  arrays.

### 8.3.3 Expression of miRNA in active sigmoid Crohn's disease

	mean	±sd	t test
miR-31	39.288	26.914	0.109
miR-494	26.268	24.374	0.171
miR-22	15.472	13.029	0.156
miR-142-5p	14.714	12.959	0.166
miR-202	13.134	14.433	0.217
miR-636	9.684	5.712	0.120
miR-451	8.653	10.015	0.233
miR-579	8.329	1.292	0.037
miR-618	7.375	5.726	0.161
let-7c	7.356	6.577	0.185
miR-32	7.074	3.988	0.135
miR-486-3p	6.860	3.907	0.102
miR-223	6.001	1.504	0.020
miR-18b	5.434	2.073	0.052
miR-29b	5.109	2.876	0.112
miR-135a	5.075	0.527	0.011
miR-15a	4.930	1.006	0.025
miR-130b	4.723	2.039	0.070
miR-487a	4.683	2.523	0.106
miR-135b	4.399	1.120	0.029
miR-224	4.190	1.701	0.066

	mean	±sd	t test
miR-548d-5p	4.169	1.257	0.042
miR-518b	4.105	0.887	0.024
miR-34a	4.077	1.247	0.041
miR-486-5p	3.899	4.142	0.250
miR-148a	3.468	1.843	0.119
miR-370	3.453	0.884	0.029
miR-20b	3.364	0.642	0.015
miR-625	3.357	2.070	0.152
miR-142-3p	3.271	2.200	0.171
miR-21	3.251	0.192	0.003
miR-455-5p	3.231	1.652	0.122
miR-652	3.196	2.203	0.182
miR-181a	3.180	1.450	0.100
miR-452	3.174	1.397	0.100
miR-18a	3.064	0.958	0.062
miR-185	2.940	0.471	0.013
miR-106b	2.902	1.305	0.106
let-7d	2.602	1.844	0.207
miR-19a	2.595	1.000	0.090
miR-182	2.538	2.064	0.240
miR-483-5p	2.533	0.361	0.012

## Chapter 8. Appendix

	mean	±sd	t test		mean	±sd	t test
miR-152	2.509	0.881	0.080	miR-101	1.455	0.545	0.223
miR-193a-3p	2.488	1.078	0.206	miR-16	1.434	1.203	0.362
miR-27a	2.438	1.061	0.117	miR-15b	1.417	0.562	0.280
miR-28-5p	2.399	0.749	0.113	miR-345	1.410	0.655	0.272
miR-29a	2.379	1.330	0.173	miR-181c	1.408	0.798	0.337
miR-140-5p	2.355	0.702	0.065	let-7e	1.389	0.953	0.355
miR-143	2.249	1.352	0.210	miR-133b	1.381	1.256	0.387
miR-99a	2.223	0.940	0.142	let-7f	1.364	0.052	0.170
miR-886-3p	2.209	1.265	0.187	miR-500	1.361	0.212	0.104
miR-885-5p	2.173	2.051	0.292	miR-323-3p	1.349	0.956	0.377
miR-147b	2.170	1.624	0.280	miR-149	1.343	0.977	0.391
miR-125b	2.166	1.214	0.187	miR-491-5p	1.341	1.184	0.388
miR-331-5p	2.144	1.143	0.237	miR-195	1.338	0.547	0.286
miR-410	2.143	0.100	0.062	miR-221	1.320	0.694	0.334
miR-19b	2.136	0.731	0.095	miR-196b	1.312	0.527	0.312
miR-132	2.131	0.327	0.050	miR-192	1.298	0.680	0.332
miR-93	2.128	0.248	0.011	miR-532-3p	1.278	0.425	0.299
miR-27b	2.121	0.849	0.124	miR-214	1.258	0.455	0.369
let-7b	2.106	1.345	0.219	miR-379	1.242	0.253	0.219
miR-660	2.093	0.927	0.143	miR-218	1.241	0.914	0.436
miR-708	2.090	0.626	0.218	miR-29c	1.234	0.541	0.389
miR-146a	2.086	0.159	0.003	miR-374a	1.229	0.352	0.263
miR-296-5p	2.028	1.563	0.262	miR-26b	1.194	0.623	0.378
miR-576-3p	2.016	0.863	0.172	miR-133a	1.186	0.865	0.415
miR-886-5p	2.006	1.028	0.202	miR-365	1.153	0.768	0.421
miR-215	2.002	1.094	0.224	miR-191	1.152	0.415	0.365
miR-193a-5p	1.975	1.509	0.273	miR-301a	1.151	0.810	0.426
miR-501-5p	1.963	0.225	0.010	miR-324-5p	1.150	0.664	0.439
miR-511	1.898	1.103	0.223	miR-539	1.145	0.182	0.390
miR-148b	1.890	1.529	0.285	miR-222	1.139	0.117	0.145
miR-150	1.876	0.486	0.079	miR-454	1.124	0.542	0.480
miR-340	1.855	1.124	0.252	miR-28-3p	1.120	0.383	0.377
miR-424	1.850	0.464	0.105	miR-335	1.118	0.862	0.477
miR-492	1.849	1.451	0.284	miR-199a-3p	1.106	0.356	0.383
miR-361-5p	1.787	2.274	0.366	miR-200b	1.080	0.132	0.339
miR-339-5p	1.785	0.039	0.005	miR-193b	1.031	0.304	0.476
miR-10b	1.746	1.175	0.284	miR-130a	1.025	0.402	0.490
miR-433	1.742	0.543	0.157	miR-342-3p	1.025	0.476	0.492
miR-103	1.728	0.793	0.195	miR-139-5p	1.023	0.365	0.497
miR-100	1.728	0.635	0.163	miR-598	1.020	0.369	0.418
miR-20a	1.709	1.087	0.263	miR-484	1.018	0.066	0.447
miR-324-3p	1.704	0.867	0.223	miR-328	1.009	0.258	0.479
miR-320	1.698	0.873	0.223	miR-671-3p	1.002	0.739	0.470
let-7g	1.682	0.702	0.185	miR-376c	1.002	0.549	0.499
miR-146b-5p	1.669	0.277	0.042	miR-330-3p	1.001	0.328	0.387
miR-127-3p	1.654	0.679	0.237	miR-30b	1.001	0.370	0.499
miR-425	1.646	0.608	0.165	miR-545	0.998	0.519	0.476
miR-532-5p	1.645	0.242	0.043	miR-339-3p	0.993	0.349	0.492
miR-155	1.637	0.357	0.094	miR-376a	0.963	0.546	0.424
miR-25	1.636	0.607	0.167	miR-203	0.941	0.125	0.342
miR-362-5p	1.625	0.992	0.270	miR-9	0.935	0.368	0.423
miR-642	1.623	0.563	0.227	miR-186	0.914	0.200	0.336
miR-590-5p	1.616	0.517	0.145	miR-301b	0.911	0.276	0.380
miR-429	1.607	0.778	0.230	miR-487b	0.900	0.593	0.430
miR-99b	1.604	0.608	0.215	miR-197	0.876	0.434	0.384
miR-106a	1.603	0.245	0.037	miR-26a	0.862	0.264	0.304
miR-194	1.588	0.746	0.234	miR-375	0.851	0.183	0.220
miR-92a	1.580	0.618	0.203	miR-331-3p	0.842	0.187	0.235
miR-628-5p	1.578	0.801	0.279	miR-30c	0.821	0.228	0.233
miR-200b	1.577	0.806	0.246	miR-338-3p	0.766	0.473	0.335
let-7a	1.567	1.211	0.332	miR-374b	0.757	0.218	0.155
miR-126	1.561	0.063	0.002	miR-190	0.748	0.324	0.237
miR-744	1.559	0.190	0.112	miR-422a	0.700	0.224	0.120
miR-145	1.552	0.809	0.270	miR-138	0.644	0.099	0.017
miR-210	1.547	0.421	0.296	miR-23b	0.626	0.254	0.160
miR-495	1.547	0.631	0.273	miR-574-3p	0.596	0.043	0.002
miR-17	1.531	0.206	0.042	miR-125a-5p	0.585	0.126	0.034
miR-140-3p	1.529	0.136	0.012	miR-134	0.584	0.228	0.122
miR-200a	1.519	0.539	0.185	miR-95	0.528	0.257	0.069
miR-10a	1.515	0.446	0.175	miR-125a-3p	0.463	0.458	0.158
miR-24	1.506	0.842	0.286	miR-1	0.446	0.196	0.027
miR-141	1.489	0.535	0.195	miR-489	0.376	0.144	0.060
miR-411	1.483	0.651	0.287	miR-204	0.348	0.245	0.084
miR-212	1.471	0.075	0.017				

Table 47. miRNAs differentially expressed in active sigmoid Crohn's disease. Microarray was performed using TaqMan® Array Human MiRNA A Cards v2.0. 188 miRNA had an expression below the threshold  $CT \leq 35$  in all array card pairs. miRNA expression was normalised to MammU6 and compared against healthy control. Microarray data is presented as mean fold difference of active Crohn's disease and analysed using the one tailed unpaired students t test.

### 8.3.4 Non expressed miRNA in active sigmoid Crohn's disease

miRNA					
miR-105	miR-219b	miR-380	miR-505	miR-520b	miR-589
miR-107	miR-220	miR-381	miR-506	miR-520d-5p	miR-597
miR-122	miR-220b	miR-382	miR-507	miR-520e	miR-615-3p
miR-124	miR-220c	miR-383	miR-508-3p	miR-520f	miR-615-5p
miR-127-5p	miR-23a	miR-384	miR-508-5p	miR-520g	miR-616
miR-128	miR-296-3p	miR-409-5p	miR-509-5p	miR-521	miR-624
miR-129-3p	miR-298	miR-412	miR-509a	miR-522	miR-627
miR-129-5p	miR-299-3p	miR-423-5p	miR-510	miR-523	miR-629
miR-136	miR-299-5p	miR-431	miR-512-3p	miR-524-5p	miR-651
miR-137	miR-302a	miR-448	miR-512-5p	miR-525-3p	miR-653
miR-139-3p	miR-302b	miR-449a	miR-513-5p	miR-525-5p	miR-654-3p
miR-146b-3p	miR-302c	miR-449b	miR-515-3p	miR-526b	miR-654-5p
miR-147	miR-325	miR-450a	miR-515-5p	miR-541	miR-655
miR-153	miR-326	miR-450b-3p	miR-516a-5p	miR-542-3p	miR-672
miR-154	miR-329	miR-450b-5p	miR-516b	miR-542-5p	miR-674
miR-183	miR-330-5p	miR-453	miR-517a	miR-544	miR-758
miR-184	miR-337-5p	miR-455-3p	miR-517b	miR-548a-3p	miR-871
miR-187	miR-33b	miR-485-3p	miR-517c	miR-548a-5p	miR-872
miR-188-3p	miR-342-5p	miR-485-5p	miR-518a-3p	miR-548b-3p	miR-873
miR-198	miR-346	miR-488	miR-518a-5p	miR-548b-5p	miR-874
miR-199a-5p	miR-34c-5p	miR-490-3p	miR-518c	miR-548c-3p	miR-875-3p
miR-199b-5p	miR-362-3p	miR-491-3p	miR-518d-3p	miR-548c-5p	miR-876-3p
miR-205	miR-363	miR-493	miR-518d-5p	miR-548d-3p	miR-876-5p
miR-208	miR-367	miR-496	miR-518e	miR-551b	miR-885-3p
miR-208b	miR-369-3p	miR-499-3p	miR-518f	miR-556-3p	miR-887
miR-211	miR-369-5p	miR-499-5p	miR-519a	miR-556-5p	miR-888
miR-216a	miR-371-3p	miR-501-3p	miR-519c-3p	miR-561	miR-889
miR-216b	miR-372	miR-502-3p	miR-519d	miR-570	miR-890
miR-217	miR-373	miR-502-5p	miR-519e	miR-576-5p	miR-891a
miR-219-5p	miR-376b	miR-503	miR-520a-3p	miR-582-3p	miR-891b
miR-219a	miR-377	miR-504	miR-520a-5p	miR-582-5p	miR-892a
					miR-96
					miR-98

Table 48. Non expressed miRNA in active Crohn's disease microarray. There are 188 non expressed miRNA including those which have no amplification and / or those with an amplification above the threshold  $CT \leq 35$ .

## 8.4 MicroRNA target prediction

The following tables contain a list of predicted gene targets for the individual miRNAs. Bioinformatic searches are limited in their ability to predict useful targets and therefore the results presented are those predicted targets that are ranked in the top 20 for TargetScan v5.2, those with a MirTarget2 score >80 and those of potential interest after review of all the targets and their known functions.

### 8.4.1 Predicted targets for miR-31

Target gene	Target scan					MirTarget2		pathway
	rank	conserved sites total	poorly conserved sites total	Total context score	Aggregate P <sub>CT</sub>	rank	target score	
RSBN1	1	2	3	-0.98	0.5	1	100	Transcription of haploid germ cells
NR5A2	2	1	2	-0.81	0.58			Role in cholesterol and bile acid homeostasis
SH2D1A	3	2	0	-0.74	0.49	9	88	Encodes a protein that plays a major role in the stimulation of T and B cells. The protein contains a SH2 domain which associates and inhibits the signaling lymphocyte-activation molecule. This protein can also bind to other related surface molecules that are expressed on activated T, B and NK cells, thereby modifying signal transduction pathways. Mutations in this gene cause Duncan disease, a rare immunodeficiency characterized by extreme susceptibility to infection with Epstein-Barr virus.
SLC1A2	4	2	2	-0.72	0.67	128	61	Clears excessive glutamate from neuronal synapses
PIK3C2A	5	2	0	-0.69	0.4	13	84	Key role in signaling pathways, proliferation, survival and migration
PRKCE	6	1	2	-0.66	0.55	28	77	Key role in TLR4 / LPS mediated signaling and INF regulation in activated macrophages (Aksoy et al., 2004).
PEX5	7	1	1	-0.6	0.32	202	52	Essential role in peroxisomal protein import.
RHOBTB1	8	1	1	-0.59	0.6	35	76	Actin and other proteins
SATB2	9	2	0	-0.58	0.54	73	69	Transcriptional regulation and chromatin remodeling, implicated in cleft palate, and other craniofacial defects in mice.
PPP1R9A	10	1	0	-0.56	0.58	47	73	No information.
DCBLD2	11	1	1	-0.54	0.31	32	77	No information.
CAMK2D	12	1	0	-0.53	0.57			Some studies in myocardial failing and endothelial barrier (in cardiac context) dysfunction, Ca signaling. Calmodulin involved in TGF-β pathway
HIF1AN	13	1	3	-0.53	0.63	88	67	miR-31 contributes to head and neck cancer by impeding HIF1AN activation (Liu et al., 2010a). Major contributor to O <sub>2</sub> homeostasis.
SLC6A6	14	1	1	-0.52	0.59	37	76	Taurine homeostasis.
STARD13	15	1	0	-0.52	0.52	148	59	Suppresses cell growth (liver cancer), inhibits activity of Raf-1-ERK1/2-p70S6K. Selectively activates CDC42 involved in TGF-β pathway.
DKFZp667G2110	16	1	0	-0.5	0.29			No information.
PAX9	17	1	0	-0.5	0.29	22	79	Mainly maxillofacial dysmorphias.
SLC5A3	18	1	2	-0.5	0.37	97	66	Mainly in coronary heart disease and diabetes observational studies. Release from fibroblasts increased by IL-1β and platelet derived growth factor together but not individually.
FGF7	19	1	1	-0.49	0.4			No information.
C2orf67	20	1	0	-0.48	0.24			Expression inhibits cardiac inflammation, hypertrophy and fibrosis (Bian et al., 2009).
CREG1	37	1	0	-0.42	0.41	12	84	Associated with lymphoproliferative disease.
SOX11	46	2	1	-0.4	0.36			No information.
CLASP2	47	1	0	-0.39	0.45	107	64	Lots of references to tumour suppression.
LATS2	59	1	0	-0.38	0.39	14	83	Required for T cell activation.
CD28	107	1	1	-0.27	0.41			Implicated in the negative control of cell growth and division.
PPP2R2A	111	1	0	-0.27	0.21	27	78	T cell signaling.
VAV3	149	1	0	-0.21	0.21			T cell activator.
PPP3CA	160	1	0	-0.2	0.21			Bind and recruit various proteins involved in the regulation of receptor protein kinases.
NCK2	191	1	0	-0.16	0.21			T <sub>H</sub> 2 pathways, crosstalk between DCs and macrophages.
TSLP	192	0	1	-0.10	<0.1			Some studies in myocardial failing and endothelial barrier (in cardiac context) dysfunction, Ca signaling. Calmodulin involved in TGF-β pathway.
CAMK2D						41	74	

Target gene	Target scan					MirTarget2		pathway
	rank	conserved sites total	poorly conserved sites total	Total context score	Aggregate P <sub>CT</sub>	rank	target score	
CDC42EP4						108	64	CDC42 is a small GTPase of the Rho-subfamily, which regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression.
CDC42SE1						119	63	As above.
HSD17B6						8	88	Oxidative and reductive activity, various effects on endogenous steroids.
IL1R1						87	67	<b>Inflammation.</b>
KIAA1967						16	82	Multiple random pathways.
LOC728054						2	100	None known.
LOC728417						5	90	None known.
MYO5A						15	83	Involved in cytoplasmic vesicular transport, implicated in several cancers
NSF						7	89	Lots of observational studies.
PARP1						141	60	Induces apoptosis.
PDZD2						17	81	Possible involvement in intracellular signaling. The protein encoded by this gene contains six PDZ domains and shares sequence similarity with pro-IL-16. The encoded protein localizes to the endoplasmic reticulum and is thought to be cleaved by a caspase to produce a secreted peptide containing two PDZ domains. This gene is up-regulated in prostate tumors.
PEX26						6	90	Interacts with PEX-1,-6 and SUFU.
SP1						132	61	<b>TGF-β pathway.</b>
TRIM67						10	86	Zink finger domain, one of many that play a role in ubiquitination.
VPS53						3	97	Vesicle trafficking in late Golgi.
ZNF74						4	92	Observational study with schizophrenia.

Table 49. Predicted targets for miR-31 using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009). Genes and pathways of particular interest are highlighted in red.

### 8.4.2 Predicted targets for miR-146b

target gene	Target scan					MirTarget2		pathway
	rank	conserved sites total	Poorly conserved sites total	Total context score	Aggregate P <sub>CT</sub>	rank	target score	
ZNF826	1	1	2	-1.19		1	99	Possible role in transcription regulation..
TRAF6	2	1	1	-0.99		2	98	<b>This protein functions as a signal transducer in the NF-κB pathway by mediating signals of the TNF receptor superfamily, members of the Toll/IL-1 family and receptors such as CD40, TNFSF11/RANCE and IL-1. This protein also interacts with various protein kinases including IRAK1/IRAK, SRC and PKCζ.</b>
NOVA1	3	2	0	-0.61		3	98	A neuron-specific RNA-binding protein, a member of the Nova family of paraneoplastic disease antigens, which are recognized and inhibited by paraneoplastic antibodies. These antibodies are found in the sera of patients with breast cancer and small cell lung cancer.
ZNRF3	4	2	0	-0.49				None known.
SIAH2	5	1	0	-0.45				Encodes a protein involved in ubiquitination and proteasome-mediated degradation of proteins. Implicated in regulating response to hypoxia
CD80	6	1	0	-0.44		109	61	<b>Macrophages in mice show increased expression, blockade of CD80 reduces colitis.</b>
LRP2	7	1	0	-0.43		194	53	Expression is induced by PPAR,
NRAS	8	1	0	-0.43		103	62	Activated in HL60 promyelocytic leukemia.
GOSR1	9	1	1	-0.38				Trafficking protein among the endoplasmic reticulum and Golgi.
USP47	10	1	1	-0.35		65	68	Has a possible role in blocking ubiquitin. viral or tumour inflammation.

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target gene	Target scan					MirTarget2		pathway
	rank	conserved sites total	Poorly conserved sites total	Total context score	Aggregate P <sub>CT</sub>	rank	target score	
GRID1	11	1	1	-0.34				Key role in synaptic plasticity, and Ca transport in the glutamate receptor ligand gated channel. Contains RNA recognition motif, has a number of functions. Interacts with PTRF (pauses transcription) and p53. A phosphoprotein phosphatase with unknown function. Activates MMP2. None known. Involved in central nervous system structure. Oncogene BCL2 is a membrane protein that blocks a step in a pathway leading to apoptosis or programmed cell death. The protein encoded by BAG1 binds to BCL2 and enhances the anti-apoptotic effects of BCL2. Found in germ cells of testes and ovaries. <b>This gene encodes the IL-1 receptor-associated kinase 1, one of two putative serine/threonine kinases that become associated with IL1R upon stimulation. This gene is partially responsible for IL1-induced up-regulation of the transcription factor NF-κβ.</b> <b>Chemokines and their receptor-mediated signal transduction are critical for the recruitment of effector immune cells to inflammation.</b> A repressor of translation by forming inactive complexes. <b>SMAD proteins are activated in response to TGF-β. The product of this gene forms complexes with other activated Smad proteins, which regulate the transcription of target genes. Mutations or deletions in this gene have been shown to result in pancreatic cancer, juvenile polyposis syndrome, and hereditary hemorrhagic telangiectasia syndrome</b> <b>ATG16 GWAS study</b> None known. None known. <b>GWAS study.</b> Involved with targeting proteins to cell membranes. Role in regulation of cell migration. Muscle function. <b>TGF-β signaling.</b> <b>Apoptosis.</b> The protein encoded by this gene interacts with atrophin-1. Major transport protein for Mg <sup>+</sup> uptake into mitochondria. Protein expression is essential for respiratory complex I and cell viability. None known. Member of the subfamily of Rho GTPases. Involved in mRNA splicing. Involved in the progression of S phase and cell survival after damage. Transcriptional repression activity. may be involved in transcription. may be involved in transcription.
SRrp35	12	1	0	-0.29		210	52	
ZNF148	13	1	1	-0.25				
PPM1M	14	1	1	-0.25				
MMP16	15	1	1	-0.22				
SNX21	16	1	0	-0.22				
ROBO1	17	1	0	-0.18				
BAG1	18	1	0	-0.17				
BNC1	19	1	0	-0.15				
IRAK1	23	1	0	-0.77		12	87	
CCBP2	27	1	0	-0.56				
EIF4G2	36	1	0	-0.42		14	86	
SMAD4	40	1	0	-0.4		18	82	
ATG12	46	1	0	-0.38				
STRBP	52	1	0	-0.37		9	89	
FBXL10	53	1	0	-0.36		11	88	
CARD10	54	1	1	-0.35				
WWC2	61	1	0	-0.32		15	86	
CDC42BPA	111	1	0	-0.09				
AMPH						197	53	
BHLHB3						115	61	
CREBL2						45	72	
ITCH						19	81	
MRS2						8	89	
PTAR1						16	86	
RHOBTB3						7	89	
SFRS6						4	91	
TIMELESS						5	91	
ZNF253						6	90	
ZNF506						13	86	
ZNF90						10	88	

Table 50. Predicted targets for miR-146b Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009). Genes and pathways of particular interest are highlighted in red.

8.4.3 Predicted targets for miR-194

Target gene	Target scan					MirTarget2		pathway
	rank	Total conserved sites	Total poorly conserved sites	Total context score	Aggregate P <sub>CT</sub>	rank	mirdb.org target score	
FBXW2	1	1	2	-0.83	0.41	6	90	Degradation of cellular regulatory proteins.
DISC1	2	1	1	-0.76	<0.1	46	79	Neurite outgrowth and cortical development, disruption of gene leads to psychiatric disorders.
TMED5	3	1	1	-0.75	0.43	23	85	None known.

Target gene	Target scan					MirTarget2		pathway
	rank	Total conserved sites	Total poorly conserved sites	Total context score	Aggregate P <sub>CT</sub>	rank	mirdb.org target score	
DCUN1D4	4	1	4	-0.74	0.36	1	94	None known.
CNTNAP2	5	1	3	-0.72	0.38	48	79	Functions on the nervous system as a cell adhesion molecule..
BTBD7	6	2	1	-0.7	0.68	86	72	None known.
ITPKB	7	1	1	-0.65	0.52	56	76	<b>Regulates immune cell function and is required for T and B cell development.</b>
HBEGF	8	2	0	-0.65	0.69	8	89	It has been shown to play a role in wound healing, cardiac hypertrophy and heart development and function.
BNIP2	9	1	1	-0.62	0.17	121	66	Plays a role with BCL-2, also an apoptotic protector.
ZFAND5	10	1	1	-0.6	0.47	146	64	None known.
MITF	11	1	2	-0.57	0.3	174	61	Role in melanocyte and osteoblast development.
FAM63B	12	1	1	-0.56	0.46			None known.
CUL4B	13	1	2	-0.54	0.48	93	71	The protein interacts with a ring finger protein, and is required for DNA replication.
CHD1	14	1	0	-0.54	0.2	92	71	CHD genes alter gene expression by modification of chromatin structure.. Associates with HDACS and RNA splicing proteins.
CROP	15	1	1	-0.54	0.51	81	73	Could be involved in the formation of spliceosome via the RE and RS domains.
KHDRBS2	16	1	0	-0.53	0.18	84	73	Member of the QKI family of genes implicated in schizophrenia, known as STAR, signal transduction of RNA.
ATP6V1H	17	1	0	-0.53	0.41	49	78	Encodes a component of vacuolar ATPase, an enzyme that mediates acidification of intracellular organelles.
ACVR2B	18	2	0	-0.52	0.75	73	74	An activin type 2 receptor. Belong to the <b>TGF-β</b> superfamily.
SETD5	19	1	1	-0.52	0.52	7	89	None known.
EPC2	20	2	0	-0.51	0.55	22	85	None known.
C7orf60	23	1	2	-0.5	0.4	10	88	ADHD genetic studies.
C14orf43	24	1	0	-0.5	0.54	14	87	None known.
C21orf91	28	1	0	-0.49	0.49	34	81	None known.
MAPK1	56	1	1	-0.41	0.5			Acts as a repressor of INF-γ induced genes. Binds the promoter of CCL5, DMP1, FIH1, IFITM1, IRF7, IRF9, LAMP3, OAS1, OAS2, OAS3 and STAT1. MAPKs regulate growth factors, differentiation, proliferation and phosphorylation of many inflammatory transcription factors. <b>Dysregulation of MAPK kinase pathways has been associated with various diseases including inflammation (p38).</b>
SLTM	59	1	0	-0.41	0.53	36	81	When overexpressed, acts as a general inhibitor of transcription that eventually leads to <b>apoptosis</b> .
BCAT1	67	1	2	-0.38	0.34			Essential for cell growth..
SUMO2	73	1	0	-0.38	0.53	16	86	Encodes a protein that is a member of the small ubiquitin-like modifier family. This protein is involved in nuclear transport, transcription, apoptosis, and protein stability.
LPHN2	162	1	0	-0.2	0.41	24	85	This gene encodes a member of the latrophilin subfamily of G-protein coupled receptors (GPCR). Latrophilins may function in both cell adhesion and signal transduction.
FOXO1	221	1	0	-0.13	0.41			<b>Apoptosis.</b>
BMP1	250	1	0	-0.02	0.36			Formation of cartilage.
CASP7						171	62	<b>Apoptosis.</b>
TSLP		0	1	-0.03	<0.1			<b>T<sub>H</sub>2 pathways</b>
CNR1						32	82	Neurotransmitter release.
MAP2						25	84	None known.
MLST8								<b>TGF-β signaling.</b>

Table 51. Predicted targets for miR-194 Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009). Genes and pathways of particular interest are highlighted in red.

8.4.4 Predicted targets for miR-200b

Target gene	TargetScan				MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	rank	mirdb.org target score	
ZEB2	1	6	0	-2.13	1	100	Interacts with receptor-mediated, activated SMADs. Represses transcription of E-cadherin.
VASH2	2	1	4	-1.5	2	100	Angiogenesis inhibitor.
LEPR	3	1	4	-1.23	52	85	LEP-R functions as a receptor for the fat cell-specific hormone leptin.
GPM6A	4	2	2	-1.06	16	96	Part of cellular wall and/ or plasma membrane.
RPS6KB1	5	1	4	-1.05	13	97	The kinase activity of this protein leads to an increase in protein synthesis and cell proliferation. Over expressed in breast cancer.
WIPF1	6	3	0	-1.04	24	93	Part of the cytoskeleton. Mutated in Wiskott-Aldrich syndrome, an X-linked recessive disorder.
MCFD2	7	1	2	-0.96	36	90	Mutations cause the combined deficiency of factor V and VIII..
ELL2	8	1	1	-0.95	4	99	The extension of an RNA molecule after transcription.
MAP2	9	1	3	-0.95	14	97	Involved in microtubule assembly, neurogenesis.
RECK	10	2	1	-0.95	5	99	Metastasis suppressor. The protein is suppressed in many tumours and cells transformed by various kinds of oncogenes. Serves as a negative regulator for MMP-9.
TRIM33	11	1	3	-0.94	6	99	A transcriptional corepressor. Interacts with TRIM24.
C16orf72	12	2	2	-0.9	17	96	Potentially down regulates transcription.
C7orf58	13	1	2	-0.89			None known.
MSN	14	2	1	-0.88	51	85	A member of the ERM protein family. ERM proteins cross link plasma membranes and actin cytoskeletons. Important for cell-cell recognition and signalling and for cell movement / adhesion.
LRP1B	15	1	1	-0.88	9	98	Belongs to the LDL receptor family, play a wide variety of roles in normal cell function and development.
CCNJ	16	2	1	-0.87	7	99	Control the progression through the cell cycle by activating cyclin-dependent kinase enzymes. Function of this cyclin is unknown.
GLT8D3	17	1	3	-0.87	81	81	None known.
FAM8A1	18	1	4	-0.86	38	89	None known.
NR5A2	19	2	1	-0.85	49	86	Plays a critical role in the regulation of development, cholesterol transport, bile acid homeostasis and steroidogenesis.
WDR91	20	1	2	-0.85	29	92	Possibly plays a role in conversion of one or more primary RNA transcripts into one or more mature RNA molecules
FAT3	21	2	2	-0.83	74	81	Plays a role in organ size through the regulation of cell proliferation and apoptosis.
SEC23A	22	1	1	-0.83	22	94	Involved in anterograde vesicle transport from ER to Golgi, Sec23 is involved in vesicle transport.
DZIP1	23	2	2	-0.83	67	83	Spermatogenesis via its interaction with DAZ.
ZNF532	24	3	0	-0.81	41	88	Transcriptional regulation.
SYNJ1	25	2	0	-0.8	27	92	Synaptic transmission and membrane trafficking.
JHDM1D	27	3	1	-0.79	75	81	Histone demethylase required for brain development.
RAB11FIP2	28	1	4	-0.79	55	84	A Rab11 effector protein acting in the regulation of the transport of vesicles from the endosomal recycling compartment (ERC) to the plasma membrane.
ZEB1	29	2	0	-0.79	3	100	Represses T cell specific IL2 gene. Represses E-cadherin, and induces an epithelial-mesenchymal transition (EMT) by recruiting SMARCA4/BRG1. Promotes tumorigenicity by repressing miRNAs
ZC3H6	30	1	4	-0.79	31	92	None known.
MKLN1	31	1	5	-0.78	25	93	Mediator of cell spreading and cytoskeleton responses to the extracellular matrix component thrombospondin 1.
LHFP	32	1	1	-0.78	21	95	Mutations result in deafness.
RNF2	33	1	2	-0.78	45	87	Transcription repression of various genes involved in development and cell proliferation.
MARCKS	34	1	1	-0.77	26	93	The protein is a substrate for protein kinase C. Involved in cell motility, phagocytosis, membrane trafficking and mitogenesis.
ZFPM2	36	2	1	-0.77	82	81	Modulates activity of GATA, important for cardiac formation.
TFAP2A	37	2	1	-0.76	83	81	Large spectrum of important biological functions including proper eye, face, body wall, limb and neural tube development.
KDR	39	1	1	-0.74	76	81	Vascular endothelial growth factor.
ERRFI1	42	2	1	-0.73	8	99	Plays a role in uterine and keratinocyte cell growth.
FEZ2	43	2	0	-0.72	70	82	Involved in axonal outgrowth and fasciculation.
GTF2E1	44	1	1	-0.72	20	95	Recruits TFIIH to the initiation complex and stimulates the RNA polymerase II.
CFL2	46	1	2	-0.71	43	87	Involved in the regulation of actin-filament dynamics.
PTPN21	50	2	0	-0.69	28	92	Member of the tyrosine phosphatase family, known to be signalling molecules that regulate cell growth, differentiation, mitotic cycle, and oncogenic transformation. Increases the activation of STAT3, but not STAT2. Studies in mice suggested the possible roles of this PTP in liver regeneration and spermatogenesis.
PCTK2	51	1	1	-0.69	10	98	May play a role in terminally differentiated neurons.

Target gene	TargetScan				MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	rank	mirdb.org target score	
WAPAL	52	2	1	-0.69	72	82	Chromosome partitioning and DNA repair
PALM2	54	2	0	-0.68	59	83	None known.
SESN1	57	1	1	-0.67	11	98	Inhibition of cell growth by activating AMP-activated protein kinase.
PHC3	60	1	4	-0.66	86	80	Role in transcriptional repression.
OSTM1	61	1	2	-0.65	30	92	Required for osteoclast and melanocyte maturation and function.
SULF1	62	1	1	-0.65	56	84	Acts as a negative regulator of TGF- $\beta$ , widely involved with inflammation. facilitates apoptosis.
ZNF652	64	1	2	-0.65	60	83	Functions as a transcriptional repressor.
SMCR7L	66	2	3	-0.65	15	97	None known.
SFRS1	69	1	3	-0.64	53	85	Member of the arginine / serine-rich splicing factor protein family, and functions in pre-mRNA splicing by binding to pre-mRNA transcripts and components of the spliceosome.
NOVA1	71	2	0	-0.63	33	91	A neuron-specific RNA-binding protein, a member of the Nova family of paraneoplastic disease antigens, which are inhibited by paraneoplastic antibodies. These antibodies are found in the sera of patients with breast cancer and small cell lung cancer.
YWHAG	72	1	1	-0.63	12	98	Important role for this protein in muscle tissue. It has been shown to interact with RAF1 and protein kinase C.
ADAMTS3	74	1	1	-0.62	61	83	The protein is the major procollagen II N-propeptidase. A deficiency of this protein may be responsible for dermatosparaxis, a genetic defect of connective tissues.
MMD	78	1	1	-0.61	77	81	This protein is expressed by in vitro differentiated macrophages but not freshly isolated monocytes.
TSGA14	80	2	1	-0.61	87	80	Cell cycle.
MED13	81	2	1	-0.61	46	87	A transcriptional coactivator complex thought to be required for the expression of almost all genes.
APOO	89	1	1	-0.58	54	85	Involved in the transport and metabolism of lipids.
LAMC1	94	2	0	-0.57	37	90	Embryonic development.
C3orf23	98	1	0	-0.56	62	83	None known.
INTS8	99	1	0	-0.56	88	80	U1 and U2 transcription.
SLC6A1	101	1	0	-0.56	34	90	Terminates the action of GABA.
RANBP10	107	3	0	-0.56	19	96	Haemostasis.
TMEM188	114	2	0	-0.55	63	83	Indirect role in intracellular lipid composition.
HRNBP3	117	1	0	-0.54	71	82	Regulates alternative splicing events.
ELAVL2	130	1	1	-0.53	47	87	RNA binding protein.
KIAA0101	136	1	0	-0.52	78	81	None known.
EGLN1	139	1	0	-0.51	57	84	Hypoxia influenced events, catalyses HIF proteins.
FBXO33	140	1	0	-0.51	39	88	Protein degradation.
GIT2	141	1	0	-0.51	50	86	GTPase-activating protein.
ERBB4	215	1	2	-0.44	73	82	Many cellular responses to ligand binding.
PLCG1	311	1	0	-0.36			T cell receptor signalling.
MAP3K1	323	1	0	-0.35			sapk / jnk, activated p38 MAPK, TLR pathways.
TOB1	502	1	0	-0.26			TGF- $\beta$ signalling.
XIAP	570	1	0	-0.23			Apoptosis.
SMAD2	603	1	0	-0.21			TGF- $\beta$ signalling.
FYN	614	1	0	-0.2			B cell receptor signalling.
PTEN	622	1	0	-0.2			Inhibitor of apoptosis.
ACVR2B	627	1	1	-0.19			TGF- $\beta$ signalling.
EP300	666	1	0	-0.17			TGF- $\beta$ signalling.
CREBBP	675	1	0	-0.16			TGF- $\beta$ signalling.
ACVR2A	725	1	0	-0.13			TGF- $\beta$ signalling.
PRKAB1	753	1	0	-0.12			Autophagy.
SP1	758	1	0	-0.11			TGF- $\beta$ signalling.
BCL2	782	1	0	-0.05			Inhibitor of apoptosis, autophagy and death receptor signalling.
MEF2D	796	1	0	0.03			Activating p38 MAPK pathways.

Table 52. Predicted targets for miR-200b Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009). Genes and pathways of particular interest are highlighted in red.

8.4.5 Predicted targets for miR-223

Target gene	TargetScan					MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	Aggregate P <sub>CT</sub>	rank	target score	
FBXW7	1	1	1	-1.36	0.86	1	99	Inactivation results in repression of TGF-β dependent transcription.
MYO5B	2	1	3	-0.85	0.56			Mutations involved in microvillous disease.
APC (adenomatous polyposis coli)	3	1	1	-0.78	0.28	9	88	Encodes a protein that acts as an antagonist of the Wnt signalling pathway. It is also involved in cell migration, adhesion, transcriptional activation, and apoptosis. Defects in this gene cause familial adenomatous polyposis.
RHOB	4	1	1	-0.73	0.51	13	87	Role in heat stress response and increased transcriptional activity of NF-κβ.
PURB	5	1	1	-0.7	0.57	56	72	Implicated in leukaemia.
SLC4A4	6	1	1	-0.69	0.45	8	89	Control of intracellular acid base balance.
FOXO1	7	1	2	-0.66	0.55	41	75	Apoptosis pathway.
ADCY7	8	1	2	-0.66	0.46	7	89	cAMP pathway
KPNA1	9	1	1	-0.65	0.41	34	77	Binds to RAG1, although outcome uncertain
ECT2	10	1	1	-0.65	0.29	22	81	Cell proliferation mainly S and M phase.
RIBC1	11	1	1	-0.64	0.43	51	72	None known.
SEPT6	12	1	1	-0.64	0.51	205	52	None known.
SLC8A1	13	1	1	-0.62	0.57	138	59	Involved in Ca channel activation.
SPTLC2	14	1	1	-0.6	0.51	118	62	None known.
SETBP1	15	1	1	-0.59	0.51	169	56	Associated with Schinzel-Giedion syndrome.
SORBS1	16	1	1	-0.59	0.54	6	89	Associated with Alzheimer's, cholesterol, blood and endocrine disorders. Proposed pathways include adherens junctions, insulin signalling pathways and PPAR signalling.
MYST3	17	1	1	-0.59	0.35	67	69	Transcriptional co-activator.
ATP7A	18	1	1	-0.56	0.55	40	75	Menkes syndrome.
SEPT10	19	1	0	-0.55	0.45	23	81	Cell cycle and division.
INPP5B	20	1	0	-0.5	0.52	105	64	Associated with disease of eye lens, and proposed function in inositol-polyphosphate 5- phosphatase activity
MEF2C	21	1	1	-0.5	0.42			Cardiac morphogenesis.
FAM46A	23	1	0	-0.48	0.39	81	67	None known.
ZNF772	24	1	0	-0.48	0.33	124	60	Response to pheromone.
ACSL3	25	1	0	-0.48	0.51	37	76	Expressed in brain. Fatty acid metabolism.
FBXO8	26	1	0	-0.48	0.47	2	91	Deletion results in growth retardation in mice.
PKNOX1	27	1	0	-0.48	0.47	39	75	Associated with Down's syndrome.
ARMCX1	28	1	0	-0.47	0.35	208	52	Tumour suppression.
SYAP1	29	1	0	-0.47	0.47			Possible involvement in liver cancer.
CENPN	30	1	1	-0.47	0.44	202	53	None known.
RRAS2	31	1	0	-0.46	0.46	30	78	The protein may function as a signal transducer. Also plays a role in activating signal transduction pathways that control cell proliferation. Mutations in this gene are associated with the growth of certain tumours.
PSIP1	32	1	1	-0.46	0.48			None known.
PARP1	34	1	0	-0.45	0.43	151	58	Differentiation, proliferation, and tumour transformation
LAYN	35	1	0	-0.45	0.4			None known.
ZXDC	36	2	0	-0.45	0.49			None known.
NUP210	37	1	0	-0.45	0.5	62	71	A structure that regulates the flow of macromolecules between the nucleus and the cytoplasm.
SP3	39	1	0	-0.44	0.47	63	70	Regulate transcription by binding to consensus GC- and GT-box regulatory elements in target genes.
ACVR2A	41	1	0	-0.43	0.47	11	88	Encodes activin A type II receptor. <b>Belong to the TGF-β superfamily.</b>
ULK2	42	1	0	-0.43	0.35	80	67	The gene is located within the Smith-Magenis syndrome region on chromosome 17
PSCDBP	43	1	0	-0.43	0.45			Expressed weakly in resting NK and T cells. The protein modulates the activation of ARF genes. This protein interacts with CYTH1 and SNX27 proteins and may act to sequester CYTH1 protein in the cytoplasm
CALML4	47	1	0	-0.41	0.47	115	62	<b>T cell receptor signalling.</b>
NFIA	52	1	0	-0.4	0.48	166	56	Transcription and replication.
LMO2	60	1	0	-0.39	0.47	3	90	Red cell development.
HSP90B1	65	1	0	-0.39	0.22	16	85	Processing and transport of proteins.
NFIB	72	1	0	-0.37	0.24			Transcription and replication.
AK2	93	1	2	-0.33	0.62			<b>Gene identified in GWAS T<sub>17</sub> cells.</b>
MEF2D	96	1	0	-0.32	0.23			Transcriptional activator in neurones and muscle.
SP1	105	1	0	-0.29	0.46			Regulates a large number of genes involved in a variety of processes such as cell growth, apoptosis, differentiation and immune responses. <b>TGF-β signalling.</b>
VAMP2	149	1	0	-0.2	0.46			<b>Associated with Crohn's disease in GWAS.</b>

Target gene	TargetScan					MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	Aggregate P <sub>CT</sub>	rank	target score	
MMP16	170	1	1	-0.16	0.58			Also targeted by miR-146b in glioma cells. Expressed by T cells.
PLA2G6	197	1	0	-0.08	0.36			Role in phospholipid remodelling, arachidonic acid release, leukotriene and prostaglandin synthesis. PLA2G2E = association found with UC in GWAS studies
CREB1						87	66	Inhibition of apoptosis.
GALNTL4						21	82	Oligosaccharide biosynthesis.
IL6ST						18	84	Forms subunit of IL-6 receptor, pro-inflammatory receptor.
LOC728709						12	87	None known.
MAP4						160	57	P38 signalling.
MPP7						10	88	Epithelial cell polarity and junction formation.
MYO5B						6	90	Possible vesicular trafficking.
PAX6						4	90	P38 signalling.
PKP4						15	85	Rho activity.
PRDM1						20	82	Maturation of B cells.
SLC7A8						14	85	Protein reabsorption in the kidney.
SLMO2						17	84	None known.
STAT1						133	60	P38 signalling.
UBR1						19	84	Pancreatic homeostasis.
TSLP						5	89	T <sub>H</sub> 2 pathways.

Table 53. Predicted targets for miR-223 Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009). Genes and pathways of particular interest are highlighted in red.

#### 8.4.6 Predicted targets for miR-375

Target gene	TargetScan				MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	rank	target score	
SLC16A2	1	1	1	-0.69	19	59	A membrane protein transporter of thyroid hormone.
ELAVL4	2	1	0	-0.50	10	67	Neuronal cells.
SHOX2	3	1	1	-0.46	13	62	Implicated in development of nervous, cardiovascular and skeletal systems. To do with hypoxic stress.
HNF1B	4	1	0	-0.39	25	56	Mutations are associated with diabetes, kidney and genital tract abnormalities, hypomagnesaemia, gout, deranged liver function and pancreas atrophy.
RASD1	5	1	0	-0.38			Interacts with rennin transcription, and signal transduction.
BAHCC1	6	1	0	-0.37	9	69	Transcriptional silencing and the remodelling of chromatin.
PTPN4	7	1	0	-0.37			A member of the protein tyrosine phosphatase family, known to regulate cell growth, differentiation, mitotic cycle, and oncogenic transformation.
GREM2	8	1	1	-0.36			Inhibits the activity of BMP, a role in organogenesis.
ZFP36L2	9	2	0	-0.36			Possible regulation of growth factors.
SYNCRIP	10	1	0	-0.35			None known.
HOXB3	11	1	1	-0.35			Involved in development and associated with AML.
ISL2	12	1	0	-0.34			Insulin. Foetal development.
FOXF1	13	1	1	-0.34			None known.
PLXNA4	14	1	1	-0.30			Forms a receptor complex with neuropilin-1 and can propagate semaphorin-3A elicited inhibitory signals into cells and neurons.
C15orf41	15	1	1	-0.30			None known.
BMPRI1B	17	1	1	-0.28			Organogenesis.
ARL4C	37	1	0	-0.19			A role in cholesterol transport.
PAX6	47	1	0	-0.18			Involved in development of eyes and nervous system.
SKI	57	1	0	-0.16			TGF- $\beta$ signalling.

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Target gene	TargetScan				MirTarget2		pathway
	rank	total conserved sites	total poorly conserved sites	Total context score	rank	target score	
ATG2B	61	1	0	-0.16			Associated with gastric and colorectal cancers <b>through autophagy</b> . None known. Regulates a large number of genes involved in a variety of processes such as cell growth, apoptosis, differentiation and immune responses. <b>TGF-<math>\beta</math> signalling</b> . None known.
NFIX	63	1	1	-0.15			
SP1	126	1	0	-0.06			
ZNF471					1	83	

Table 54. Predicted targets for miR-375 Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009). Genes and pathways of particular interest are highlighted in red.

## 8.4.7 Predicted targets for miR-422a

Target gene	TargetScan				MirTarget2		pathway
	rank	total conserved targets	total poorly conserved targets	Total context score	rank	target score	
PHC3	1	1	1	-0.41	93	62	Some references to osteosarcoma and bones.
RBMS3	2	1	0	-0.41			Liver fibrosis.
SULF1	3	1	0	-0.41	36	71	Involved in cell signalling. Heparan sulphate 6-O-endosulfatases, such as SULF1 activity modulates the effects of heparan sulphate by altering binding sites for signalling molecules. <b>Acts as a negative regulator of TGF-<math>\beta</math>, widely involved with inflammation. Facilitates apoptosis.</b>
VANGL1	4	1	2	-0.37	13	81	Membrane proteins, neural tube defects.
PLEKHG2	5	1	0	-0.34			Associated with leukaemia.
GOLT1A	6	1	0	-0.31			Associated with hepatoblastomas. Hypertension.
SLC39A9	7	1	0	-0.3			Membrane transport protein, 1 of 400 in family to do with metal transport.
DYRK1A	8	1	1	-0.27			<b>Increased expression improves survival in sepsis, by abrogating sepsis induced lymphocyte apoptosis.</b>
PAX8	9	1	0	-0.24			Transcription factor for the thyroid-specific expression of the genes exclusively expressed in the thyroid cell type, maintaining the functional differentiation of such cells.
SFRS3	10	1	0	-0.2			May be involved in RNA processing in relation with cellular proliferation and/or maturation.
GLS	11	1	0	-0.17			Catalyzes the first reaction in the primary pathway for the renal catabolism of glutamine.
BCL2L2	12	1	1	-0.12			<b>Apoptosis.</b>
TSLP		0	1	-0.06			<b>T<sub>H</sub>2 pathways</b>
DSCAM					10	83	Neuronal development.
ELAC1					3	89	tRNA maturation.
FOXP1					6	87	Brain development.
ITPRIPL2					4	89	None known.
KDSR					12	81	Catalyst.
LOC100128178					5	87	None known.
MGC13057					1	92	None known.
NKX3-1					7	87	Transcriptional repressor. Prostate development.
NR2C2					11	82	Transcription.
PAPOLA					2	91	Polymerase.
RPN2					8	84	N-oligosaccharyl transferase enzyme.
SLC7A6					15	81	Neural amino acids.
TRAF6					67	65	<b>TLR pathway.</b>
UHRF1					9	84	Transcriptional regulation.

Table 55. Predicted targets for miR-422a Using TargetScan v 5.2 and MirTarget2 v4.0. Pathways are taken from NCBI Gene database (Pruitt et al., 2009). Genes and pathways of particular interest are highlighted in red.

## 8.5 Centrifuge calculations

Experimental procedures were carried out and described using the rpm settings on all centrifuges. The table below gives the approximate conversion as calculated using the manufacturers technical data, (Eppendorf, 2012, Kendro laboratory products, 2001).

	Rotations per minute (rpm)	Gravitational force (g)
Centrifuge 5417R, FA-45-24-11, (eppendorf, UK)	13,000	15,600
	2,000	370
	1,500	200
Centrifuge 5810R, A-4-62, (eppendorf, UK)	1,500	450
	2,000	800
Centrifuge Sorvall®	2,000	800
Legend T (Kendro Laboratory Products, Germany)	1,500	400

## 8.6 Statistical calculations

### 8.6.1 Mann Whitney calculations for section 4.4

	Control (n)	Active UC (n)	Inactive (n)	Active vs control (U value)	Inactive UC vs control (U value)
miR-31	21	25	19	55	42
miR-146b	21	25	19	39	74
miR-194	21	20	11	49	113
miR-200b	19	25	19	49	71
miR-223	21	25	19	47	34
miR-375	21	25	19	38	151
miR-422a	21	25	19	30	96

### 8.6.2 Mann Whitney calculations for section 4.5.1

#### 8.6.2.1 miR-31 in Ulcerative Colitis

Statistical differences between median values calculated with two tailed Mann Whitney and shown as U values, p values

Active UC			
	5-ASA (n = 7)	Azathioprine (n = 10)	Infliximab (n = 2)
No treatment (n = 10)	20, p = 0.198	8, p = 0.001	7, p = 0.711
5-ASA (n = 7)	-	9, p = 0.005	6, p = 0.889
Azathioprine (n = 10)	-	-	1, p = 0.061

Inactive UC			
	5-ASA (n = 10)	Azathioprine (n = 10)	Infliximab (n = 2)
No treatment / control (n = 8)	34, p = 0.317	12, p = 0.071	6, p = 0.711
5-ASA (n = 10)	-	10, p = 0.016	9, p = 0.909
Azathioprine (n = 10)	-	-	1, p = 0.143

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### 8.6.2.2 miR-31 in Crohn's disease

Statistical differences between median values calculated with two tailed Mann Whitney and shown as U values, p values

Active Crohn's Disease		
	Azathioprine (n = 4)	Infliximab (n = 4)
No treatment (n = 3)	0, p = 0.025	0, p = 0.014

Inactive Crohn's Disease	
	Azathioprine (n = 5)
No treatment / control (n = 3)	3, p = 0.062

### 8.6.3 Mann Whitney calculations for section 5.3

Statistical differences between median values calculated with two tailed Mann Whitney and shown as U values, p values

Mucosal lymphocytes			
	Control (n)	Active UC (n)	Active vs control (U value, p value)
miR-31	4	4	1, p = 0.029
miR-146b	4	4	2, p = 0.114
miR-194	4	4	0, p = 0.014
miR-200b	4	4	0, p = 0.014
miR-223	4	4	0, p = 0.029
miR-375	4	4	3, p = 0.100
miR-422a	4	4	3, p = 0.100

## 8.7 The effect of PHA on cell lymphocyte culture

Stimulation with PHA has a differential effect on leukocyte subtypes. In a mixed leukocyte sample, monocytes and granulocytes decline over time, CD3<sup>+</sup> cells increase, and CD4<sup>+</sup> and CD8<sup>+</sup> cells decrease until a nadir on day 1, then normalise by day 2 and proliferate thereafter (O'Donovan et al., 1995).

Short term cell disruption and possible death may contribute to the miR-31 and TSLP expression levels; therefore cell death was quantified by staining cells with Trypan blue.

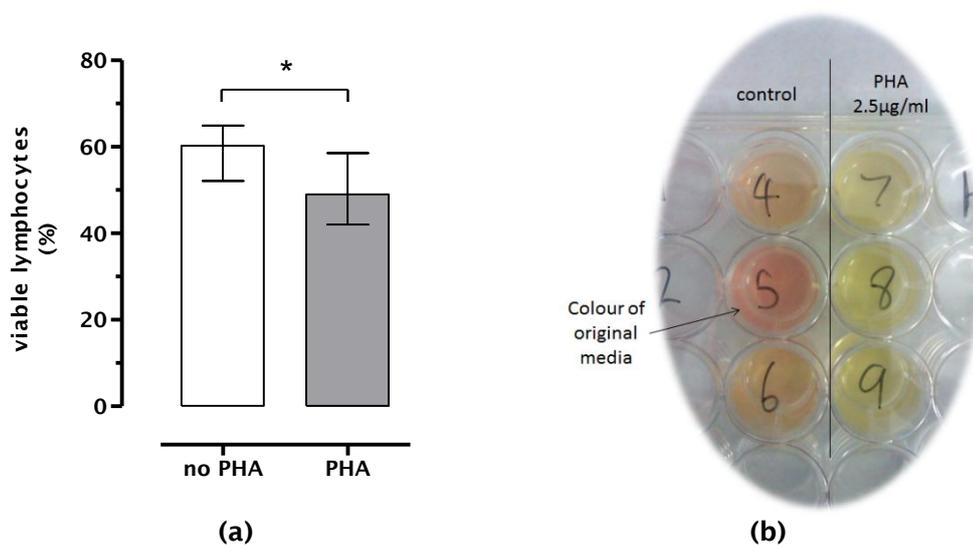


Figure 55. Lymphocyte viability following PHA culture. (a) % lymphocyte viability measured by trypan blue staining. (b) The effect of PHA stimulation on well conditions after 48 hours culture. HT29 cells were cultured at  $0.5 \times 10^6$  cells per well. Median values are compared with one tailed Mann Whitney U test.\*  $p \leq 0.05$ . Whiskers are IQR.

After 48 hours incubation, visual inspection of the culture media showed that in the stimulated wells (labelled in Figure 55b as numbers 7, 8 and 9) the media had turned yellow indicating a reduced pH. The two causes of a reduced pH; metabolic activity and cell death are differentiated by counting viable cells. The median number of viable lymphocytes is 60.2% to 49.0% in unstimulated and stimulated cells respectively,  $p = 0.020$ . However despite this reduction in cell viability the house keeper gene in the RT-qPCR was similar between the groups, maintaining good Ct values (17 to 19), indicating preservation of gene transcription.

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**Despite the 11% reduction in cell viability, gene levels did not match this change, with an increased miR-31 expression of 510% and a decreased TSLP mRNA expression of 29%.**

**The purpose of this section is to show how culture affected well conditions and the necessity to change culture medium regularly. It also indicates the increased cell activity in the stimulated wells.**

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## **Chapter 9. Bibliography**

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