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Mapping the interleukin-23 regulated transcriptome in ulcerative colitis

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MAPPING THE INTERLEUKIN-23 REGULATED TRANSCRIPTOME IN ULCERATIVE COLITIS

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Dedication

I dedicate this work to my wonderful and patient wife Natalie

Whilst drafting this thesis I appreciated that I was following in the footsteps of Sir Samuel Wilks and Sir William Hale-White's by researching ulcerative colitis at Guy's Hospital which was a wonderful and unexpected discovery. I so pleased to be able to continue to study ulcerative colitis in the same location that they first described classified it.

Quote

“A good idea is about ten percent and implementation, hard work and luck is 90 percent”

Guy Kawasaki, 1980s Apple employee and Silicon Valley entrepreneur

“Perfect is the enemy of good”

Voltaire, French philosopher 1694 - 1778

Table of contents

Contents

Table of figures

from lamina propria mononuclear cells from patients with active ulcerative colitis cultured with standard media +/- IL-23 303

Abstract

Inflammatory Bowel Disease (IBD) is undergoing a transformation with an expanding repertoire of drugs targeting various aspects of the immune response. In recent years, we have witnessed the emergence of three novel classes of drugs that target leukocyte trafficking to the gut (vedolizumab), antibodies that neutralize key cytokines (ustekinumab) and inhibitors of cytokine signalling pathways (tofacitinib). IL-23 is considered a key cytokine in IBD, and drugs targeting its specific p19 subunit are in advanced development. Insights into how IL-23 might regulate mucosal immune cells are now needed.

There is also a pressing need to develop precision medicine approaches since all agents assessed so far are hampered by primary and secondary loss of response. It is desirable to develop personalised strategies to inform which patients should be treated with which drugs. Stratification by clinical parameters alone lacks sensitivity, and alternative modalities are now needed to deliver precision medicine in IBD.

The rapid decrease in cost of whole tissue transcriptomics combined with increased expertise in bioinformatics has permitted the use of complex tools to analyse RNA like never before. In this thesis I have investigated the use of transcriptomics to probe the biological impact of IL-23 signalling in UC. Particular focus was given on

the transcriptional effects of IL-23 in ex vivo cellular models using colonic tissue from. diseased colons of UC patients.

We created two models namely whole colonic biopsy and lamina propria mononuclear cells (LPMCs) using tissue from active ulcerative colitis and exposed biological replicates to IL-23 or standard culture media and then performing RT-qPCR and RNAseq. We then identified statistically differentially expressed genes from the IL-23 exposed compared to non-exposed samples. Using Ingenuity Pathway Analysis permitted us to identify relevant up and downregulated pathways and upstream regulators to enhance the meaning of our discovery of differentially expressed genes.

We aimed to:

1. identify the IL-23 responsive genes, pathways, and functions in UC
2. establish whether these IL-23 responsive genes were upregulated in active ulcerative colitis in reposited datasets
3. determine whether IL-23 responsive genes were able to impact response to UC therapies

IL-23 stimulation of LPMCs from active UC orchestrated a strong pro-inflammatory transcriptional programme with significant upregulation of IL-22 and IFNγ by RT-qPCR.

As expected IL-23 induced the Th17 pathway but also induced novel pathways such as HMGB1, cancer and angiogenesis and promotes cell trafficking and chemotaxis, previously unheralded functions. Network analysis revealed IL-17A, IL-17F, IL-22 and IFNG appear to function as central nodes involved in the coordination of the inflammatory process.

By utilising publicly available reposited data sets from active colonic tissue from patients with UC, we were able to interrogate these samples for enrichment of our gene sets and with allied clinical data we could judge the effectiveness of our gene signature to predict response to therapies. The IL-23 LPMC enrichment scores were statistically significant higher in active disease.

We had access to the UNIFI dataset courtesy of Janssen. The IL-23 LPMC gene signature showed statistically significantly higher enrichment scores in a number of endpoints including endoscopic healing and clinical remission at week 8. Furthermore, stratification by enrichment scores increased the likelihood of achieving endpoints and was further enhanced by including CRP in the analysis. For example, unstratified patients had 12.7% chance of meeting the endpoint of clinical and endoscopic remission but when IL23 LPMC enrichment score <0 and CRP <5 22.8% of patients achieve this endpoint. We also showed that the IL23 LPMC enrichment score was

statistically different in patients who received placebo and who responded, which highlights an important group who may not require therapy at all.

Surprisingly, enrichment scores were not higher in anti-TNF non-responders in available reposited datasets. Utilising data from the PURSUIT trial (152 patients of golimumab in UC) data courtesy of MSD – the IL-23 LPMC signature had statistically higher enrichment scores in patients who did not achieve week 6 endoscopic remission but did not show a difference in other endpoints. We showed that by stratifying enrichment scores we can improve the chance of predicting response such as 18% if enrichment score <0 achieved clinical remission and endoscopic healing vs 12% if unstratified. Furthermore, when combined with CRP <5 the proportion can be increased further to 20%.

The analysis of colonic explants has raised more questions than answers them. We found that IL-22 was significantly upregulated when investigated with RT-qPCR but relevant pro-inflammatory pathways such as the Th17 pathway or T cell activation were not found. Instead, we found activation of IBD relevant oxidative phosphorylation ad PPARγ pathways and downregulation of aryl hydrocarbon receptor pathways.
Together these pathways do not form a coordinated pro-inflammatory effect of IL-23 as expected but create a series of opposing effects.

The colonic explant signature showed higher enrichment scores in active UC though showed reduced enrichment in anti-TNF non-responders contrary to the hypothesis.

We demonstrate the generation a gene signature by exposing biological replicates of active colonic tissue from patients with UC to IL-23 and identifying the differentially expressed genes. We demonstrate that the colonic explant model was not successful in inducing a coordinated pro-inflammatory response but the lamina propria experiment induced appropriate and expected Th17 cytokines as well as novel findings suggestive of co-activation with IL-1 cytokines. Furthermore, we demonstrate the IL-23 LPMC signature can differentiate active from inactive UC and healthy controls and is statistically significantly more expressed in patients achieving clinical endpoints when receiving anti-IL12p40 antibodies in UNIFI trial. We show through stratification of the enrichment scores and addition of biomarkers we can further improve the rates of achieving endpoints. We have shown the potential of a transcriptomic based gene signature biomarker in UC though further work will be required to improve the sensitivity and to ratify our results before it can be utilised in a clinical setting.

Acknowledgements

I started at Guy's & St Thomas' Trust (GSTT) in October 2014 as a Registrar in Gastroenterology hoping to gain experience in IBD. In January 2015 mid way through a particularly tricky sigmoid, Jeremy Sanderson asked me if I wanted to be a research fellow. I jumped at the chance, and he suggested I go to see Nick Powell to formulate a research project.

So, I went to Guy's to see Nick and was immediately struck by Nick's incredible enthusiasm for IBD and research. His office was a tip, pieces of paper lay everywhere, and he was always losing pens but on the spot he produced 5 different projects, told me I should apply for a grant from ECCO and gave me about 10 papers to read. A little overcome I got cracking with writing the grant though I understood little Nick guided me through it and to the surprise of us all was awarded €30,000 by ECCO. Throughout Nick has pushed me to author papers, inspired me with innovative ideas and has encouraged me the whole way and I thank him deeply for this. Often we would meet at his house in the evening and discuss issues over a glass of wine as we lived only 3 roads away in Balham. This had the disadvantage though of being first in line to feed his cats whilst he was on holiday.

I could have had my vengeance however after a lab Christmas party as we stumbled home we thought we would have a last pint at our local in The Bedford in Balham. We got to the bar and started chatting to Barry (over 6 foot Irish traveller bare knuckle boxer who lost half his fights by knockout) and another man and woman. When asked how we knew each other I responded, 'Nick is my boss.' Barry's face turned purple "I do not like bosses. Do you want me to do him in?" I hesitated for a moment then downed our pints and scarpered home quickly.

In October 2015 I started as a part time IBD Fellow at GSTT and part time research on the 5th floor at Guy's. I thought I had the perfect job – half time being an IBD fellow at one of the best IBD centres in the country dealing with complex patients and the exciting world of biologics whilst learning a new craft of laboratories and 'wet' science. However, this was a far harder mix then I thought it would be. Clinical work never really stops and the inevitable pull back to the wards to cover a clinic or an endoscopy list meant that the lab time suffered at times. By dropping clinical sessions and focusing more on the lab research this balance became easier but if I had my time again I would dedicate more time to the lab as a block as I underestimate how much can be learnt by sitting next to a 'real' scientist and the benefits of immersing oneself in the research.

Soon after starting as a research fellow, I met Nat Prescott who became my second supervisor. Nat has this amazing air of calm and has always made time to explain process qPCR techniques or statistical methods. She kindly allowed me to utilise her lab to learn how to extract RNA from samples and was helped greatly by Laura Demandt, her PhD student, and Ariella, Research Assistant in the lab.

Esperanza Perucha is a wonderful person and a fantastic mentor and scientist. I credit her for restarting my ailing lab technique as well as inspiring a passion for precision and statistics. It is fair to say that my lab work would have produced no meaningful results had I not worked with Espe, so I owe her my deepest thanks. Furthermore, her bright disposition, permanent smile and gallows humour on Brexit never failed to amuse me.

Polychronis Pavlidis is a lovely chap. He taught me so much about data processing, R, manipulation of data as well as how to work unbelievably hard. Furthermore, he taught me much about Thessaloniki, his grandmother's sausages, and the issue of Macedonia. He is a great friend who will go far in the IBD world, and I hope to be there when he rises to the top. He is the epitome of a hardworking, altruistic, and kind doctor and is a true inspiration to all clinician scientists.

Umar Niazi led the team of bioinformaticians who also included Sanjana Sood and Domenico Cozzetto who so kindly analysed the RNAseq on behalf of the Biomedical Research Centre based at Guy's. Their professional and engagement in their clinically minded approach meant that we received high quality results with true clinical relevance which is so important in translational research.

Tsui Tjir-Li worked extremely hard as a lab assistant and was instrumental in processing the large amount of RNA that we produced. His exact and methodical approach was perfect for the labour intensive, multi-process steps that are required to produce high quality RNA.

Zain Kassam was instrumental in the collection of patient samples from endoscopy and would accompany me to the Tuesday morning endoscopy list weekly so ensure the correct pots were used in the right order etc. I would like to think he learnt a bit about endoscopy too! His aim was to go to medical school, and I hope that wish comes true for him as I think he would be an exceptionally good doctor particularly given his background in basic science.

Laura Constable works hard and is very driven in her goal to get her PhD. Whilst we did not work directly together on a project, she was a delight to sit near to in

the office and was always available to pick her brain about a RT-qPCR or gripe that I had. I am enormously proud that I bought her first ever gin and tonic.

John Lo is one of the calmest people I know. His ability to rise above the clamour and get on with the task is almost unrivalled. I have tried hard to be more like John in my day-to-day life and is a constant inspiration to me.

Amy Day introduced me to lab work back in 2015 and her patience whilst I made numerous mistakes was legendary. Her help in those early days were invaluable and without her basic skills teaching I would have struggled. Her mentorship and perseverance was very helpful, and I want to thank her deeply for this.

Robin Dart is a very natural clinical scientist and oozes confidence, and ideas flow easily from his brain down into his work. We collaborated on a few small projects, and he was kind enough to teach me the grid technique which was pioneered in the lab in which he worked. I owe him much gratitude as this technique saved and enhanced many of experiments. Furthermore, we had some great nights out notably in Washington DC, where we ate many oysters.

Natalie, my wonderful wife, has driven me forward and encouraged me to complete this thesis more than anyone. She has seen the hours I have spent sitting at the kitchen table and is an amazing sounding board for all my gripes and irritations involved in the analysis and writing of this document. She has spent many hours supervising Emilia whilst I worked on the thesis so without her support, I suspect it would never have been written. I dedicate the thesis to her.

Emilia, my beautiful daughter, I am sorry that I have spent so much time drafting this thesis rather than playing with you. However, long-term I hope this inspires you to work hard and persist in your aims.

My Mum, Laura, is non-medical and non-scientific but gladly answered the call to read through and note any spelling or grammar mistakes. Given my distinct lack of ability to spell this was invaluable. Few parents would do this for their child but as always my mum stepped up, like she always does, and did a brilliant job. Thank you Mum.

The final thank you should do to COVID-19 and the Victorian government of Australia. A strange thank you but due to COVID-19 surge of cases in Victoria, Australia where we lived for a year whilst I was an IBD fellow at the Alfred Hospital

in Melbourne, we endured 5 months of the harshest lockdown in the world where we were only allowed outside for 1 hour a day for exercise. Therefore, instead of enjoying exploring the city, sitting in vineyards in the Yarra Valley or Mornington Peninsula I spent many hours drafting this thesis.

Declaration

I declare that the work presented is my own. I contributed to study conception and design, applied successfully for funding, recruited patients, collected, and processed samples, performed experiments, collected, amalgamated, and analyzed data, prepared figures, and authored this thesis.

Contributions from other members of the Nick Powell group or other parties are clearly acknowledged throughout this thesis.

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European Crohn's & Colitis Organisation (ECCO) grant in 2016 for €30,000 for consumables

Part time clinical fellowship at Guy's & St Thomas' Hospitals provided a stipend

Publications & presentations arising from thesis

Interrogating host immunity to predict response to therapies in Inflammatory Bowel Disease
Digby-Bell J, Monteleone G, Atreya R, Powell N.
Nat Rev Gastro Hep 2020

The transcriptomic signature of IL-23 treated laminar propria mononuclear cells is significantly enriched for genes in the Th17 pathway and is enriched in active UC

Poster ECCO 2019

Abbreviations

AhR	Aryl hydrocarbon receptor
AUC	Area under the curve
BRC	Biomedical Research Centre
CD	Crohn's Disease
CDAI	Crohn's Disease activity index
cDNA	copy DNA
CLE	Confocal Laser Endomicroscopy
DEG	Differential Expressed Genes
DNA	deoxyribonucleic acid
DDW	Digestive Diseases Week
ECCO	European Crohn's and Colitis Organisation
ELISA	Enzyme-linked immunosorbent assay
fdr	false discovery rate
FPKM	fragments per kilobase of exon model per million reads mapped
GvHD	Graft versus Host Disease
GWAS	genome-wide association study
HBSS	Hanks' Balanced Salt Solution
HC	Healthy Control

IBD	Inflammatory Bowel Disease
IFN	Interferon
IL	Interleukin
IPA	Ingenuity Pathway Analysis
JAK	Janus Kinase
LPMC	lamina propria mononuclear cell
MAPK	mitogen activated protein kinases
NAD	Nicotinamide adenine dinucleotide
OSM	Oncostatin M
OSMR	Oncostatin M receptor
PCA	principal component analysis
PPAR	Peroxisome proliferator-activated receptor
QC	quality control
qPCR	quantitative polymerase chain reaction
RIN	RNA integrity score
RNA	ribonucleic acid
RNAseq	RNA sequencing
RT	room temperature
RXR	retinoid X receptor
SD	standard deviation

SOP	standard operating procedure
STAT	signal transducer and activator of transcription
TNF	tumour necrosis factor
UC	Ulcerative Colitis
UCEIS	Ulcerative Colitis Endoscopic Index of Severity

1. Introduction

1.1. Ulcerative Colitis- an overview

Ulcerative colitis (UC) is part of a wider family of diseases known as Inflammatory Bowel Disease (IBD) which comprises 2 main forms: namely UC and Crohn's disease (CD). UC solely affects the colon of the gastrointestinal tract and is a chronic inflammatory disorder (Danese and Fiocchi 2011) which cause severe symptoms of diarrhoea, rectal bleeding and abdominal pain, a deterioration of mental health and considerable decrease in quality-of-life (Knowles, Graff, et al. 2018; Knowles, Keefer, et al. 2018). Moreover, the impact on patient's personal wealth due to lower productivity has been described (van der Valk et al. 2014). Total economic burden of UC has been estimated at $8.1-14.9 billion annually in the United States and at €12.5-29.1 billion in Europe; total direct costs were $3.4-8.6 billion in the United States and €5.4-12.6 billion in Europe (Cohen et al. 2010).

1.2 History of UC

UC was first described by Sir Samuel Wilks, a prestigious physician at Guy's Hospital in 1859 when he suggested that UC be considered in a different category from specific epidemic dysentery (Wilks 1859a).

Furthermore, Wilks describes one of the earliest cases of CD in the post mortem of a woman believed to have been murdered by her husband in the wonderfully titled paper 'Morbid appearances in the intestine of Miss Bankes', a title which today would not be permitted due to current ethical guidelines on confidentiality (Wilks 1859b).

The term 'ulcerative colitis' was first used by Sir William Hale White, another Guy's Hospital physician, in a case series which differed this disease entity from "growth, dysentery, tubercle, typhoid and so forth." (White 1909)

160 years is a long time in medicine and diagnosis and treatment of UC has clearly advanced more than could be imagined but there are still challenges with poor response to treatment, side effects and surgery still being required. To place this into context, albeit not a fair one as UC is a far more complex disease, HIV was first

described in 1981 (Gottlieb et al. 1981) and within 40 years it is considered a chronic illness with patients expected to have a normal life expectancy (Trickey et al. 2017).

1.3 Incidence and prevalence

Prevalence of UC in the UK is estimated to be 240/100,000 with about a third of diagnoses being made in patients less than 25 years old (Kaplan and Ng 2016). Current hypotheses suggest that UC incidence is increasing over time in developed countries such as the UK but will be considerably increased in developing countries such as China (Kaplan and Ng 2016). Figure 1.3 below summarises the increased incidence of UC diagnoses globally.

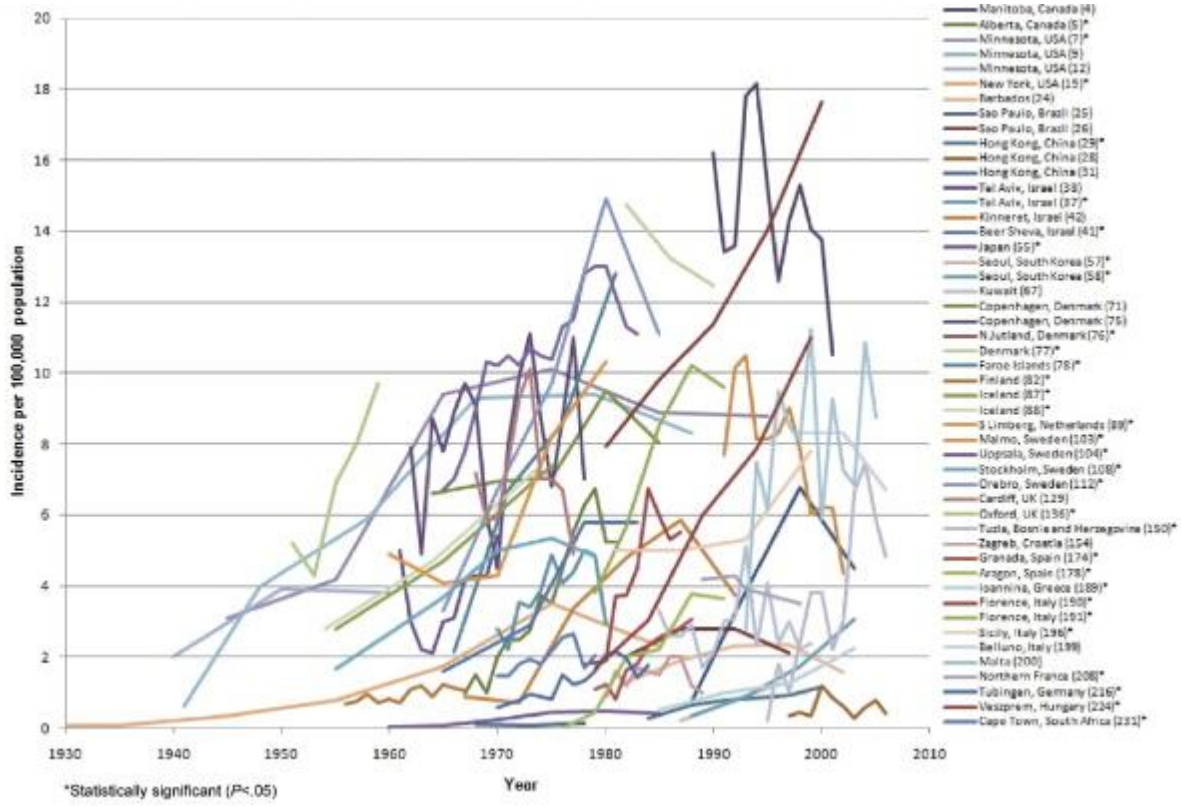


Figure 1.3 temporal trends of incidence of UC diagnoses across a number of countries with multiple country based studies with statistically significant increases in incidence over time (Molodecky et al. 2012)

1.4 Clinical features of UC

UC only affects only the colon in contrast to CD which can affect any part of the GI tract (Cosnes et al. 2011). UC is characterised by a relapsing and remitting mucosal inflammation resulting in significant and often debilitating symptoms (Cosnes et al. 2011). Symptoms of UC are classically of diarrhoea, rectal bleeding, and urgency. Abdominal pain is normally a feature of Crohn' s disease rather than UC. Up to 15% of patients will present with severe disease requiring inpatient treatment. Symptoms can be wide ranging and vary depending on the distribution and severity of the disease but can vary from asymptomatic to debilitating diarrhoea requiring hospital admission. Three quarters of patients report that their UC affects their work, and two thirds affects their leisure activities (Ghosh and Mitchell 2007).

Extra-intestinal manifestations are present in approximately a third of patients (Vavricka et al. 2011) with the most common being peripheral arthritis. Notably primary sclerosing cholangitis and pyoderma gangrenosum are more common in UC than CD (Vavricka et al. 2011).

Long term complications of UC include an increased risk of colorectal cancer (CRC) (Ekbom et al. 1990; Beaugerie et al. 2013). Risk factors for CRC have been identified as having extensive disease and being diagnosed at a young age (Ekbom et al. 1990).

Surveillance endoscopy has been shown to be beneficial in that diagnosis of early CRC and dysplastic lesions have increased and interval and advanced CRC has decreased over time (Choi et al. 2015).

Surgery is not uncommon in UC with up to 15.6% of patients requiring major abdominal surgery in the first 10 years after diagnosis (Frolkis et al. 2013). However, surgery is dramatically less than the rates of surgery in CD from the same centres (46.6%). Interestingly surgery rates for both UC and CD have reduced when compared to the previous six decades (Ahmad et al. 2018) though the reasons for this are not clear and likely to be multifactorial including transition of care from surgery to gastroenterology, better medical treatments and improved awareness of active disease through endoscopic surveillance.

Mortality of patients with UC is not greater than that of the general population (Cosnes et al. 2011).

1.4.1 Endoscopic features of UC

UC diagnosis is based on endoscopic and histological findings. Endoscopic findings are summarised in figure 1.3.1. below but include loss of vascular pattern, erosions, and bleeding. The severity of disease can be judged endoscopically using a Mayo score

(Schroeder, Tremaine, and Ilstrup 1987) or a UCEIS (UC Endoscopic Index of Severity) (Travis et al. 2012) score.

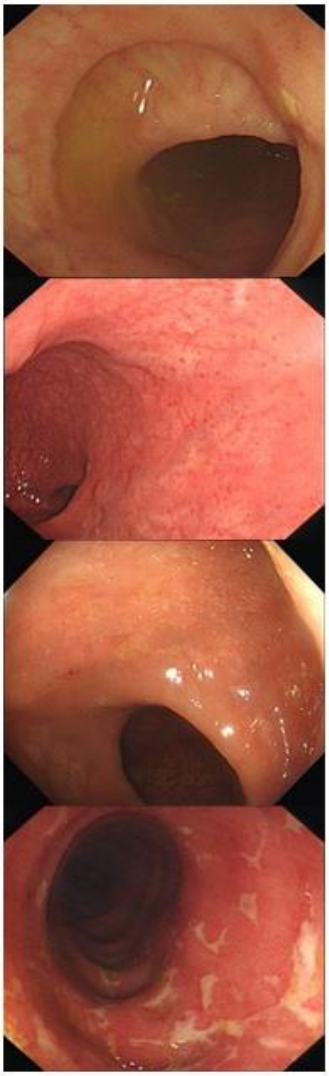


Image A - Mayo 0

Image B - Mayo 1. partial loss of vascular pattern and mucosal erythema

Image C - Mayo 2. Complete loss of vascular pattern and erosions

Image D – Mayo 3. Mucosal erythema and ulceration

Figure 1.4.1. Endoscopic appearances of UC as stratified by the Mayo endoscopic score. Mayo 0 - normal mucosa, Mayo 1 - mildly active disease, Mayo 2 and 3 - active disease. (Images created by author)

1.4.2. Histological findings

Histological characteristics are of mucosal inflammation with crypt abscesses, crypt distortion, inflammatory cells in the laminar propria as shown below in figure 1.3.2.

A number of validated histological scoring systems exist with the most extensively used in clinical being the Gebboes Score which comprises scoring of 6 descriptors (Geboes et al. 2000) with good reproducibility. However, given the complexity of the index and therefore the time taken to report by histopathologists it is rarely used in clinical practice. The Nancy index uses 3 descriptors and classifies disease into 5 categories (0- inactive, 4- severely active) and has high intra and inter reader correlation (0.88 and 0.86 respectively) (Marchal-Bressenot et al. 2017). This simpler scoring system is now used in many IBD centres with the benefit of easier comparison of longitudinal samples.

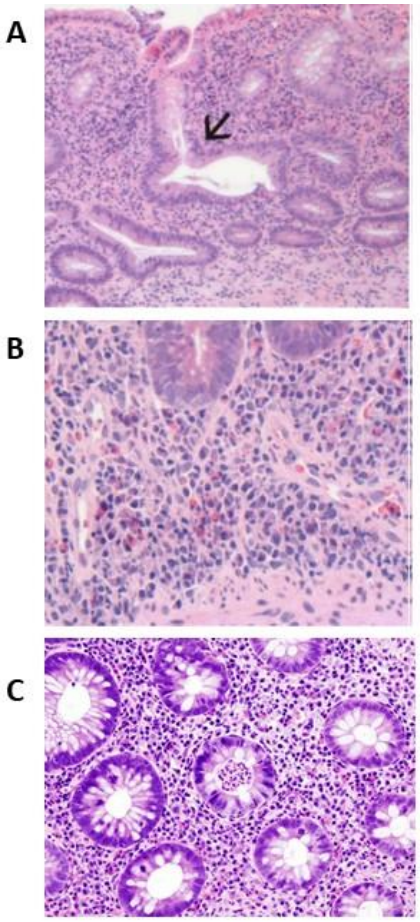


Figure 1.4.2. Histological features of UC (Feakins 2014)

A - crypt architecture distortion

B – increase in plasma cells in the laminar propria

C – crypt abscess

1.4.3. Classification of UC

The Montreal classification (figure 1.3.3.) (Satsangi et al. 2006) was created as it was recognised that patients may have a different disease course dependent on the distribution of disease and may benefit from differing treatments e.g., proctitis and left sided disease would benefit from topical treatments more than pancolitis. This classification is summarised in an image below. However, the Montreal classification for UC has its drawbacks most notably it does not allow for progression or regression of disease over time (Magro et al. 2012).

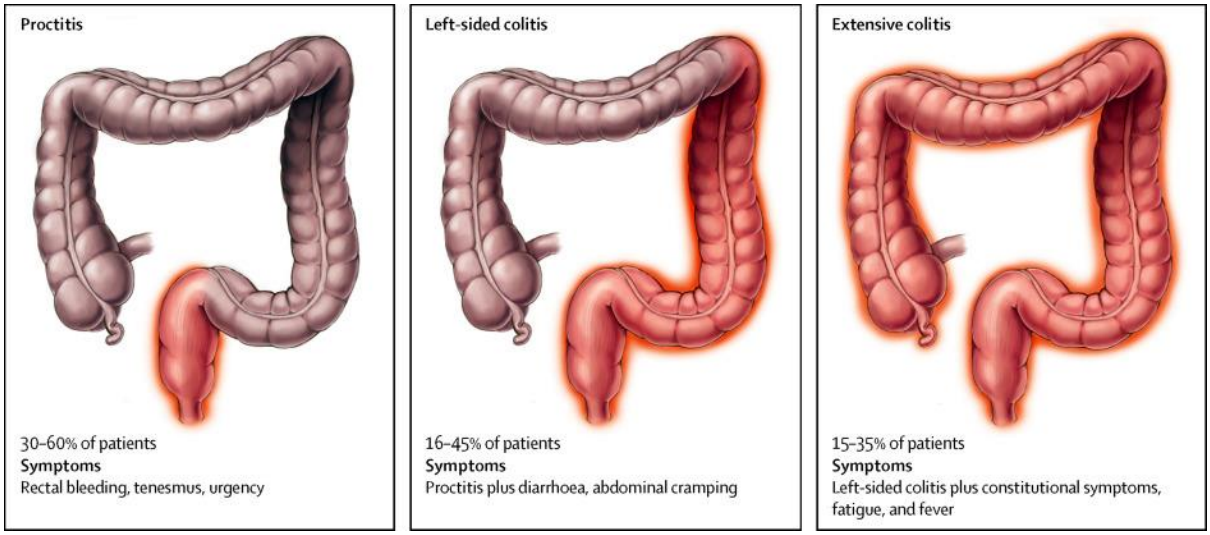


Figure 1.4.3. Summary of the Montreal classification of UC (Ungaro et al. 2017)

1.5 Immune mechanisms of disease in IBD

Mechanistic insights into IBD pathogenesis have been achieved in numerous ways. Reductionist approaches in pre-clinical IBD models have helped to identify functionally important mediators, most often cytokines, with indispensable roles in initiation and progression of disease. Moreover, utilizing pre-clinical models validated the administration of anti-inflammatory cytokines to abrogate IBD and the administration of cytokine antibodies to prevent or reduce the incidence of IBD (Powrie et al. 1994).

Multiple cell types have been identified as key to the pathogenesis of IBD. Barrier alterations by injury due to environmental factors, drugs or infection permits commensal translocation and exposure to the innate immune system namely macrophages, dendritic cells, and intestinal epithelial cells (IEL) which lead to a rapid and effective inflammatory response against microbial invasion. Furthermore, via cytokine production and antigen presentation can lead to specific activation of the adaptive immune system via (Choy, Visvanathan, and De Cruz 2016). In contrast to the innate immune system the adaptive immune system produces highly specific responses to the antigen. For example Th1 cells produce IFN-γ, TNF-α and IL-12 (Romagnani 1994) whereas Th17 produces IL17A, IL-17F, IL-21 and IL-22 and therefore enacting different pro or anti-inflammatory effects upon the mucosa(Zhou et al. 2007).

Validation of inflammatory pathways in human disease is challenging and often relies on demonstrating increased expression of different mediators in IBD patients in comparison with subjects without disease. Altered patterns of cytokine production in IBD were first noted in 1990s for example the elevation of IL-6 and TNF in patients with IBD compared to controls (Mitsuyama, Sata, and Tanikawa 1991; Breese et al. 1994). Further supporting evidence from large scale genetic susceptibility studies including Genome Wide Association Studies (GWAS) has helped to corroborate target identification and has implicated novel targets for example STAT1, STAT3, STAT4, CC-chemokine receptor 6 (CCR6), CC-chemokine ligand 2 (CCL2), CCL13, IL-12 receptor (IL-12R), IL-23R and Janus kinase 2 (JAK2). Further studies have identified IBD risk loci that contain genes that encode cytokines (for example, IL-2, IL-21, interferon-γ (IFNγ), IL-10 and IL-27) thus highlighting a potentially major role for these cytokines in disease pathogenesis (Jostins et al. 2012; Liu et al. 2015; de Lange et al. 2017).

Patients with extreme IBD phenotypes, such as very early onset disease associated with monogenetic disorders (e.g., *IL10R* deficiency) (Glocker et al. 2009; Kotlarz et al. 2012), have provided new insights into pathways required for maintenance of intestinal homeostasis.

Together these different approaches have culminated in the identification of numerous pro and anti-inflammatory pathways (see Figure 1.5.1. (Neurath 2014)) and hence

identification of druggable candidate molecules and pathways, many of which have been subsequently targeted in clinical trials with either monoclonal antibodies or small molecule inhibitors (see Figure 1.5.2.).

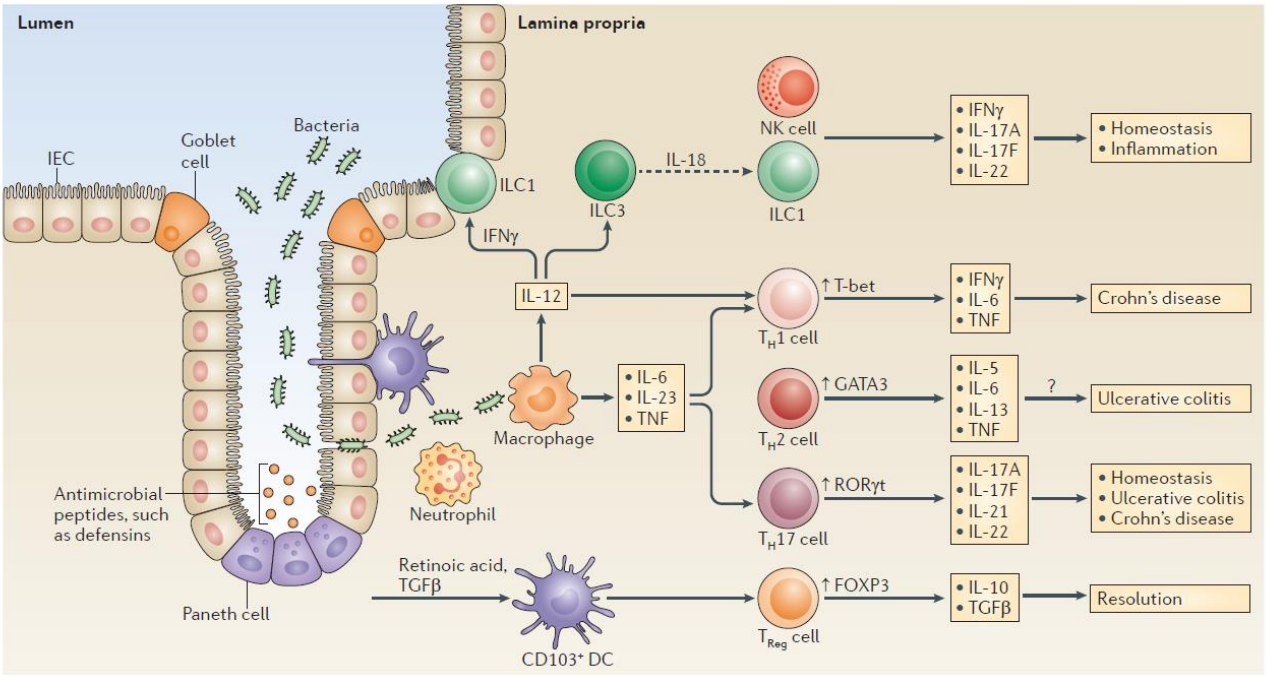


Figure 1.5.1. In patients with inflammatory bowel disease (IBD) pro-inflammatory and anti-inflammatory cytokines have been shown to be produced by various cells of the mucosal immune system. In particular, dendritic cells (DCs), neutrophils, macrophages, natural killer (NK) cells, intestinal epithelial cells (IECs), innate lymphoid cells (ILCs), mucosal effector T cells epithelial cells (IECs), innate lymphoid cells (ILCs), mucosal effector T cells (T helper cells (Th1, Th2 and Th17)), regulatory T (TReg) cells produce cytokines in the inflamed mucosa. The key transcription factors and cytokines produced by T helper cell subsets in IBD-affected mucosa are shown. GATA3, GATA-binding protein 3. (Neurath 2014)

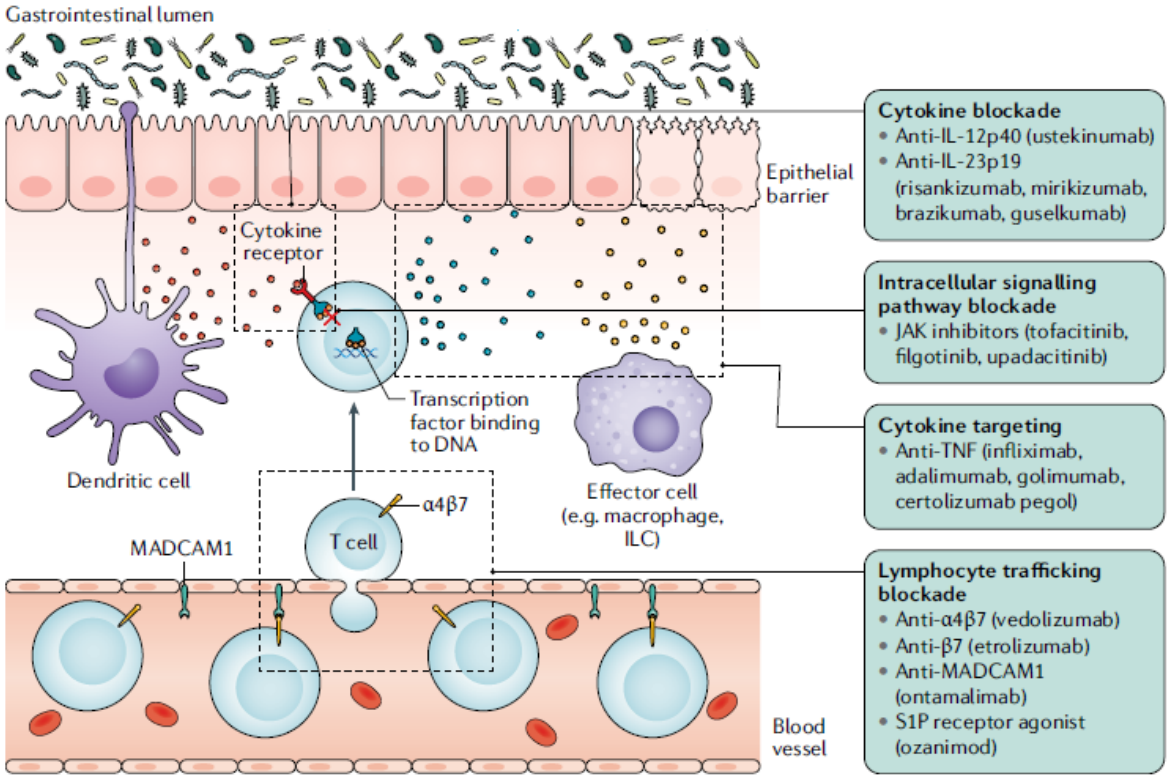


Figure 1.5.2. Drug targets in inflammatory bowel disease (IBD) can be broadly separated into: (a) cytokine receptor blockade (e.g. IL12p40: ustekinumab, IL-23p19: risankizumab, mirikizumab, guselkumab and brazikumab), (b) T-cell intracellular signalling pathway blockade (JAK inhibitors: tofacitinib, filgotinib, upadacitinib), (c) cytokine targeting (anti-TNFα: infliximab, adalimumab, golimumab, anti-IL17: secukinumab, anti-IL6: PF-04236921) (d) lymphocyte trafficking blockade (anti-α4β7: vedolizumab, anti-β7: etrolizumab, anti-MADCAM-1: ontamalimab, S1P receptor antagonist: ozanimod) (Digby-Bell et al. 2020)

1.5.1 The pivotal role of TNF in the immunopathogenesis of IBD

The pleiotropic cytokine tumor necrosis factor (TNF) is a crucial mediator of mucosal inflammation(MacDonald et al. 1990). TNF expression is markedly increased in the serum and intestinal tissue of IBD patients (Breese et al. 1994). It stimulates inflammation in multiple ways including activation and proliferation of immune cells (Scheurich et al. 1987; Hurme 1988; Ranges et al. 1988; Yokota, Geppert, and Lipsky 1988), induction of cytokine and chemokine production(Israel et al. 1989; Liu and Han 2001), stimulation of chemotaxis(Ming, Bersani, and Mantovani 1987; Wedemeyer et al. 1999), angiogenesis and extracellular matrix degradation(Pender et al. 1998; Okuno et al. 2002). Depending on which receptor it ligates, TNF may either promote intestinal epithelial cell apoptosis (via TNF receptor (TNFR) 1(Garrett et al. 2007)) or render T cells resistant to apoptosis (via TNFR2). It is initially synthesized as a monomeric transmembrane precursor protein (mTNF) on immune cells (e.g., macrophages, monocytes, and lymphocytes) and nonimmune cells (e.g., fibroblasts, endothelial cells). The soluble trimeric form (sTNF) yielded via proteolytic cleavage by the matrix metalloprotease TNF-converting enzyme (TACE)(Black et al. 1997). Both TNF forms exert their biological activity by binding to two structurally distinct transmembrane TNFRs on target cells. While TNFR1 is constitutively expressed on most cell types and preferentially binds to sTNF, TNFR2 expression is tightly regulated and shows much higher affinity for mTNF (Wajant, Pfizenmaier, and Scheurich 2003). mTNF not only acts

as a ligand, but also as a receptor when engaged by TNFRs, thereby transmitting intracellular signals to mTNF bearing cells, in a process termed outside-to-inside (reverse) signaling (Slevin and Egan 2015).

It is likely that mTNF, rather than sTNF, plays a dominant role in IBD. In an experimental transfer colitis model, mice unable to generate soluble TNF because of a non-cleavable mutant TNF gene develop severe colitis (Corazza et al. 2004). Specific neutralization of mTNF but not of sTNF alone also ameliorates disease in pre-clinical models(Perrier et al. 2013). Furthermore, mTNF overexpression in intestinal macrophages leads to activation of TNFR2 bearing mucosal T cells, bolstering their resistance to apoptosis. TNFR2 overexpression by T cells results in aggravated experimental colitis and its absence attenuated experimental colitis in another mouse model (Holtmann et al. 2002).

This is further corroborated by a case report of an extreme IBD phenotype in a child with very early onset IBD-like chronic small intestinal inflammation and skin disease. The patient, born from consanguineous parents, was found to harbor a loss of function deletion of ADAM17, which encodes TACE, an enzyme responsible for cleaving mTNF to liberate sTNF. The patient had marked impairment of sTNF production, and presumably constitutive overexpression of mTNF and enhanced TNFR2 signaling which likely contributed to early onset gut inflammation (Blaydon et al. 2011).

The ultimate proof of the key role played by TNF in IBD pathogenesis is highlighted by the efficacy of anti-TNF mAbs demonstrating significantly improved outcomes compared to placebo in UC (infliximab (Rutgeerts et al. 2005; Sandborn et al. 2009), adalimumab (Reinisch et al. 2011; Reinisch et al. 2013), golimumab (Sandborn, Feagan, Marano, Zhang, Strauss, Johanns, Adedokun, Guzzo, Colombel, Reinisch, Gibson, Collins, Jarnerot, et al. 2014)) and CD (infliximab (Hanauer et al. 2002), adalimumab (Colombel et al. 2007), certolizumab (Sandborn et al. 2007)). However, the soluble TNF receptors etanercept (Sandborn et al. 2001) and onercept (Rutgeerts et al. 2006) failed in clinical trials in CD, consistent with the therapeutic mechanism of anti-TNF agents extending beyond simple neutralization of sTNF. Current paradigms indicate that the main mechanism of action of anti-TNF mAbs is induction of T cell apoptosis (Atreya et al. 2011). Intestinal T cell resistance to apoptosis is important for sustaining chronic intestinal inflammation (Billmeier et al. 2016; Slevin and Egan 2015) and induction of apoptosis in mTNF bearing target cells is a central anti-inflammatory mechanism mediated by anti-TNF agents in IBD. Rapid induction of T cell apoptosis occurs in the mucosa of IBD patients responding to anti-TNF therapy(Atreya et al. 2011), and in a study visualizing apoptotic intestinal immune cells by single-photon emission computed tomography and labeled annexin V application, there was a significant correlation between the induction of mucosal cell apoptosis and therapeutic efficacy of initiated anti-TNF therapy (Van den Brande et al. 2007). Furthermore, the lack of

efficacy of etanercept in CD has been attributed to its reduced ability to induce apoptosis in mucosal T cells, which might be explained by hampered binding capacities to mTNF. The induction of T cell apoptosis is believed to be induced by reverse signaling of mTNF-bound anti-TNF agents, leading to activation of the caspase 8-mitochondrial permeability pathway, or inducing caspase 3-dependent apoptosis (Billmeier et al. 2016; Slevin and Egan 2015). Further studies indicate that therapeutically effective anti-TNF antibodies induce T cell apoptosis by binding to mTNF expressing CD14+ macrophages, thereby blocking the mTNF/TNFR2 co-stimulation pathway between $CD14^+$ macrophages and $CD4^+$ T cells in the lamina propria (Atreya et al. 2011). Based on these observations, harnessing techniques to quantify mTNF bearing cells in diseased mucosa is a conceptually attractive way of demonstrating whether this is likely to be an important pathogenic mechanism in an individual patient, and of course whether anti-TNF is likely to be effective.

1.5.2 Cytokine driven immune networks

Cytokines play a significant role in driving inflammation and tissue injury in IBD.

Although there are many similarities in cytokine responses in CD and UC, there are also major differences. CD is a canonical Th1-mediated disease (Parronchi et al. 1997)..

Some studies indicate that Th2 responses are expanded in UC (Heller et al. 2005),

although other investigators have challenged this paradigm (Biancheri et al. 2014). In both CD and UC, there is an expansion of the Th17 cells (Rovedatti et al. 2009; Kobayashi et al. 2008).

1.5.2.1 IL-23

IL-23 was first reported by Oppmann *et al.* in 2000, who discovered a p19 protein that formed a covalently linked complex with the IL-12p40 subunit, which was functionally different to IL-12 (Oppmann et al. 2000).

IL-23 plays a key role in the regulation and maintenance of the $CD4^+$ Th17 lineage and promotes the production of IBD relevant cytokines IL-17, IL-22 and IFNγ (Annunziato et al. 2007) (Liang et al. 2006). Interestingly, IL-23 does not perform this role directly by promoting Th17 cell differentiation as naive T cells owing do not express IL-23R (Bettelli et al. 2006). T cell receptor engagement in combination with specific environmental conditions and cytokine signals (including transforming growth factor-β (TGFβ), IL-1 and IL-6) induces a network of transcription factors with retinoid related orphan receptor-γt (RORγt) as the master regulator that promotes expression of the prototypic Th17 cytokine IL-17A and IL-23R (Ivanov et al. 2006). Interaction of IL-23 with its receptor then activates STAT3, which acts to promote transcription of IL-23 R and RORγt, building up a positive feedback loop that stabilizes gene expression required for Th cell activation and effector functions.

Furthermore, IL-23 has also been shown to effect other important drives effector function of ILCs and acts on other important immune regulators such as natural killer cells and γδ T cells (Buonocore et al. 2010; Geremia, Arancibia-Carcamo, et al. 2011).

These master regulator cells play orchestrate intestinal inflammation and IL-23 plays a critical part in activating them.

Large scale genome wide association studies (GWAS) studies have identified the specific receptor for IL-23 (IL-23R) as a susceptibility gene for IBD. (Jostins et al. 2012; Duerr et al. 2006; Cho and Brant 2011). Indeed, it was found to be the second largest effect size of any IBD locus, second only to NOD2 (Jostins et al. 2012).

In preclinical models and human IBD, IL-23 stimulates production of pro-inflammatory cytokines by mucosal ILCs (Powell et al. 2015; Krausgruber et al. 2016; Geremia, Arancibia-Cárcamo, et al. 2011; Powell et al. 2012). IL-23 has also been shown to induce IL-17, a pathogenic cytokine, in CD4+ lamina propria cells from UC surgical specimens (Kobayashi et al. 2008). Crucially, blockade of IL-23 is highly effective in preclinical models of IBD (Powell et al. 2012; Chen et al. 2006).

Ultimate proof of the key pathogenic role in ulcerative colitis comes from randomised control trials of anti-IL12p40 antibodies (ustekinumab) and anti-IL23p19 antibodies (risankizumab, and mirikizumab) discussed further in 1.6.4.

In health, IL-23 plays a role in the host response to pathogens. Mice lacking expression of either IL-23p19 or the IL12pp40 are unable to clear *Citrobacter rodentium* after oral

challenge and succumb to lethal infection (Mangan et al. 2006). Moreover, when mice deficient in IL12p40 were exposed to *Mycobacterium tuberculosis* infection, they demonstrated increased bacterial growth, reduced production of IFN-gamma, and had increased compromised to mice lacking IL12p35 subunit expression (Cooper et al. 2002)

1.5.2.2 IL-12

IL-12 plays an important role in the differentiation of Th1 cell (Trinchieri 1994), and also promotes production of inflammatory cytokines by activated T cells (Monteleone et al. 1999; Parronchi et al. 1997). IL-12 stimulates effector cytokine production by an important population of mucosal resident innate immune cells, called innate lymphoid cells (ILCs) (Goldberg et al. 2015; Artis and Spits 2015)

1.5.2.3 IL-12 / IL-23 axis

IL-23 and IL-12 are heterodimeric cytokines. They share an IL-12p40 subunit. IL-12 couples with IL-12p35, and IL-23 couples with IL-23p19. Binding of IL-12p35 to its receptor IL-12Rβ2 or IL-23p19 to IL-23R results in structural alterations that facilitate

high-affinity association of the IL-12p40 subunit with the IL-12Rβ1 chain. These processes induce the activation of JAK2 and TYK2, which are associated with both receptors. Activation of the IL-12 receptor complex results in phosphorylation and homodimerization of signal transducer and activator of transcription 4 (STAT4), whereas IL-23 receptor signaling results in the formation of STAT3 and STAT4 homodimers. STAT3 and STAT4 translocate into the nucleus where they activate distinct transcriptional programmes (Moschen, Tilg, and Raine 2019). Summarised in figure 1.5.2.3.

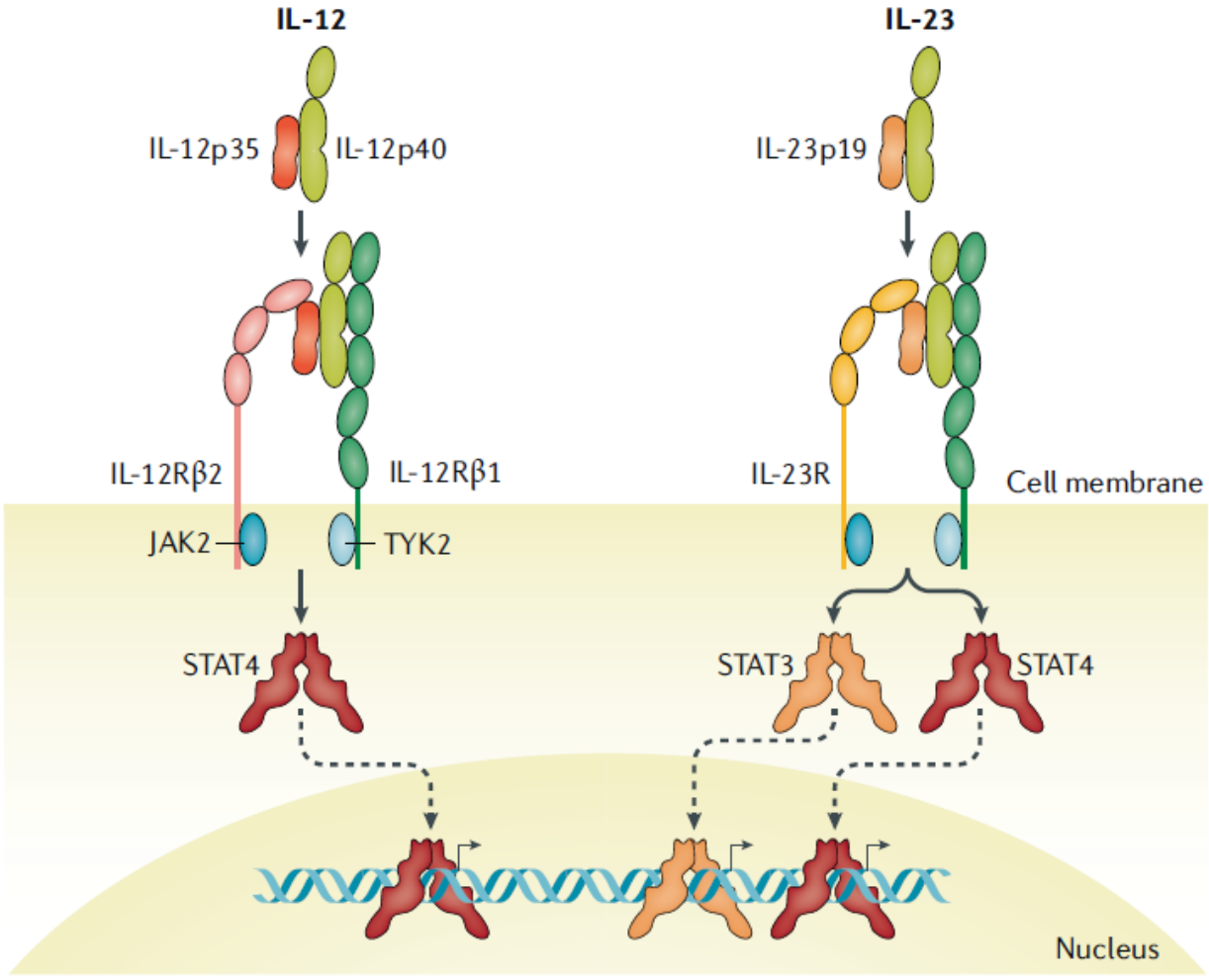


Figure 1.5.2.3. Structure and signalling of IL-12 and IL-23 (Moschen, Tilg, and Raine 2019)

Together these observations led to the hypothesis that targeting IL-12 and/or IL-23 may be effective in IBD. Targeting the p40 subunit, which is common to both IL-12 and IL-23 receptors was considered an especially attractive approach, since it may simultaneously impact both Th1 and Th17 lineages. Furthermore, it was established that in a phase II study that p40 blockade was associated with a greater response rate compared to placebo (Mannon et al. 2004). This led to the development of ustekinumab, an anti-IL12p40 mAb, which has been shown to be effective compared to placebo in CD and UC (Feagan et al. 2016; Sands, Sandborn, et al. 2019) and is now licensed for CD and UC in the UK and across the globe.

However, the story is more complicated than initially envisaged. Blockade of the IL12 specific subunit p35 has no impact on colitis in pre-clinical models (Uhlig et al. 2006), suggesting that the effectiveness of p40 blockade is due to IL-23 blockade rather than IL-12 blockade. These findings have directed attention to the possible advantages of selective IL-23 blockade in IBD. Selectively targeting the p19 subunit, which is unique to IL-23, is highly effective in ameliorating colitis in pre-clinical models (Hue et al. 2006; Powell et al. 2012).

Intriguingly, anti-IL23p19 therapy is more effective than anti-IL12p40 therapy in psoriasis with 90% or greater reduction in the Psoriasis Area and Severity Index (PASI) score of 77% (64 of 83 patients) for risankizumab (90-mg and 180-mg groups pooled),

as compared with 40% (16 of 40 patients) for ustekinumab ($p<0.001$) (Papp et al. 2017). If comparable results are observed in IBD, selective IL-23 blockade could be a 'game changer' in the field of IBD treatments. 2 studies are currently recruiting in CD comparing the efficacy of ustekinumab to a IL23p19 molecule. Furthermore the GALAXI study is a phase III programme comparing 3 doses of guselkumab, ustekinumab and placebo (ClinicalTrials.gov) and is due to report in 2023. The SEQUENCE study is comparing ustekinumab and risankizumab in patients with CD who have failed anti-TNF is currently recruiting and expected to complete recruitment in 2023 (ClinicalTrials.gov).

However, these studies leave some questions open. For instance, it remains unclear why p19 blockade should work better than p40 blockade given that both these treatments suppress IL-23 function. There may be pharmacokinetic explanations (e.g., ustekinumab underdosing) as well as biological explanations. One hypothesis is that IL-12 has regulatory effects, so ameliorating the anti-inflammatory effects of IL-23 blockade. This could involve cross regulation of different T-cell lineages (e.g., Th1 cells suppress other T-cell lineages such as Th17 cells therefore supressing Th1 cells with p40 blockade would lead to "escape" of Th17 cells). Another possibility is that p19 blockade has higher affinity than p40 blockade resulting in a more complete IL-23 suppression of function.

1.5.2.4. JAK inhibitors

IBD-relevant cytokines exert their function by binding to their specific receptors, which then transduce signals to target cells through recruitment and activation of janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways (O'Shea and Plenge 2012; Coskun et al. 2013). JAKs constitutively bind to the inactive cytokine receptors and are activated in response to the binding of the cytokine to its receptor. Activated JAKs phosphorylate multiple sites on the cytokine receptor thereby allowing binding for STAT molecules (Villarino et al. 2015; Yamaoka et al. 2004). Once phosphorylated, STATs dimerize and translocate to the nucleus, where they regulate transcription of many target genes. Inhibition of JAK/STAT molecules offers the advantage of suppressing multiple cytokine pathways simultaneously. Tofacitinib, is a small molecule inhibitor of JAK1 and JAK3 and, therefore inhibitor of all STAT molecules. Although lacking efficacy in CD (Panés et al. 2017), tofacitinib is effective for induction and maintenance therapy in UC compared to placebo (Sandborn et al. 2017) and is now licensed by the FDA and EMA. The reason tofacitinib is effective in UC but not in CD remains elusive. It is conceivable that the lack of efficacy in CD may in part rely on the fact that inhibition of JAK/STAT molecules might suppress not only inflammatory signals but also anti-inflammatory mechanisms, since JAK/STAT

molecules are also involved in the IL2-mediated maintenance of Tregs and IL10-driven Treg-mediated immunosuppression (Goldstein et al. 2016; Chinen et al. 2016; Chaudhry et al. 2011).

1.5.3 Lymphocyte trafficking blockade

Leukocyte recruitment to the intestine is mediated by interactions between integrins and cognate endothelial ligands which include members of the immunoglobulin superfamily of adhesion molecules, vascular cell adhesion molecule-1, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and intercellular adhesion molecule-1 (Hamann et al. 1994; Postigo, Teixido, and Sanchez-Madrid 1993). For example, T cell recruitment to the gut is mediated by interactions between the integrin heterodimer α4β7 expressed by the T cell, and MAdCAM-1 expressed by the endothelium of post-capillary venules supplying the gut (Berlin et al. 1995). Monoclonal antibodies targeting α4β7 (vedolizumab) are effective in CD (Sandborn et al. 2005; Sandborn et al. 2013) and UC (Feagan et al. 2013) demonstrating significantly improved outcomes compared to placebo.

A small molecule inhibitor of Sphingosine-1-Phosphate (S1P), which regulates the egress of lymphocytes from lymph nodes into the systemic circulation and thereby limits the accumulation of lymphocytes in inflamed gut, are efficacious in phase III studies (Sandborn et al. 2021) and are now in use in clinical practice.

1.6 Treatment of UC

Treatment of UC is dependent on the disease distribution (proctitis, left sided disease or extensive), severity of disease (mild, moderate, severe requiring hospitalisation which is best assessed by the Truelove and Witt criteria (Truelove and Witts 1954)), age of the patient, disease course, current and previous medications, side effects from previous medications and extra-intestinal manifestations.

Treatment targets have changed in the past 30 years markedly. Previous aim was to achieve clinical (i.e. symptom free) remission however through the analysis of clinical trials endoscopic remission as identified as a key endpoint which maintains clinical remission and resection free survival (Neurath and Travis 2012). Moreover, treat to target regimes have been developed such that treatment changes dependent on objective clinical markers e.g., faecal calprotectin which has been associated with better endoscopic outcomes (Colombel et al. 2018). Even though the CALM study was in CD parallels can be drawn in the treatment of UC.

1.6.1 Aminosalicylates

Aminosalicylates (also named mesalamine, mesalazine, 5-ASA and 5-aminosalicyclic acid) are used as a first line therapy in UC for mild to moderate disease (Harbord et al. 2017). Their application may be via the oral or topical (rectal) route with the options of suppositories or enemas. These modes of delivery allow delivery of the drug targeted to the distribution of the disease.

In proctitis a suppository should be first line as the distribution of drug is dependent on the volume such that a lower volume preparation (e.g. suppository) will have a higher concentration in the rectum than that of a larger volume preparation (e.g. enema) (van Bodegraven et al. 1996). With the same knowledge left sided disease should be treated with an enema preparation.

Cochrane review has shown that topical mesalazines are more effective than placebo at inducing clinical, endoscopic, and histological remission (Marshall et al. 2010; Wang et al. 2016). In a meta-analysis rectal mesalazine delivery has been shown to induce remission as well as oral mesalazine preparations (Ford et al. 2012) though combined oral and rectal preparations are better than either option on their own (Ford et al. 2012). Mesalazine rectal preparations have been found to be superior to corticosteroid

rectal preparations (Marshall and Irvine 1997). Furthermore, a combination of rectal mesalazines and corticosteroids has been found to be more effective than either agent alone (Mulder et al. 1996). Once daily dosing is as effective as split dosing and though not shown to improve efficacy is likely to do so outside of the trial setting (Wang et al. 2016). Furthermore, there is no evidence that one mesalazine preparation has improved efficacy or safety than other preparations (Wang et al. 2016). Escalated dosing up to 4.8g mesalazine induces remission quicker than lower dose mesalazine (median cessation of 9 days with 4.8g v 16 days with 2.4g) (Hanauer et al. 2005).

1.6.2 Corticosteroids

Regardless of distribution of disease corticosteroids are indicated to induce clinical remission if mesalazines have been insufficient, not tolerated, or contraindicated (Harbord et al. 2017). Exact length of time a clinician should wait to see if mesalazine is going to induce remission is unclear if a patient's symptoms deteriorate, or rectal bleeding persists beyond 10–14 days, or sustained relief from all symptoms has not been achieved after 40 days of appropriate 5-ASA therapy, additional therapy with oral systemic steroids should be started (Harbord et al. 2017; Lichtenstein et al. 2007; Kamm et al. 2007).

1.6.2.1 Systemic corticosteroids

Systemic steroids e.g. prednisolone are effective in inducing clinical remission in UC (Lennard-Jones et al. 1960; Truelove, Watkinson, and Draper 1962) with starting doses of <15mg and for a duration of < 3 weeks associated with early relapse or non-response (Baron et al. 1962). Despite these seminal studies dating from the 1960s they still have a valid role in UC treatment today. Whilst useful as induction agents, systemic corticosteroids should not be used as long-term therapy due to considerable side effects such as diabetes, osteoporosis, weight gain, mood disorders (Mowat et al. 2011).

1.6.2.2 Gastrointestinal release corticosteroids

Of the gastrointestinal release corticosteroids there is good evidence for modified release budesonide (budesonide MMX, Cortiment) with CORE I and II studies showing statistically significant increased proportion of patients reaching a combined clinical and endoscopic remission at week 8 over placebo (17.4 v 4.5%, OR 4.49; 95% CI 1.47 to 13.72; p=0.0047 (Sandborn et al. 2012; Travis et al. 2014). Though there was no statistical difference with budesonide MMX and mesalazine 3g/day treatment. However non-MMX budesonide (ileal release preparations) have no evidence to support its use in UC (Gross et al. 2011). Beclomethasone has been little investigated in UC with only 5 studies identified in a meta-analysis but it appears to be well tolerated

and efficacious when compared to aminosalicylates (Manguso et al. 2016) and can be considered a valid alternative to systemic corticosteroids (Harbord et al. 2017).

1.6.2.3 Parenteral corticosteroids

Hydrocortisone or methylprednisolone intravenously are the treatments of choice for acute severe UC (Truelove and Jewell 1974; Rosenberg, Ireland, and Jewell 1990). They are given for 5-7 days and on day 3 an assessment should be made using the Travis criteria (patients with more than eight stools on that day, or a stool frequency between three and eight together with a CRP > 45 mg/l) (Travis et al. 1996) to decide if the patient is at high risk of a colectomy and if so to decide if a rescue therapy of ciclosporin, tacrolimus or infliximab is indicated dependent on current and previous medications (Harbord et al. 2017).

1.6.3 Immunomodulators

1.6.3.1 Thiopurines

Mercaptopurine was first found to be effective in UC in 1962 (Bean 1962). Following this initial case report several uncontrolled cohort studies have shown benefit with azathioprine (Fraser, Orchard, and Jewell 2002; Lobo et al. 1990). Large, randomised control trials for thiopurines in UC are missing and most evidence for its use is based on meta-analyses. The meta-analysis by Khan *et al* suggests that in quiescent UC,

there were three trials involving 127 patients and there was a statistically significant benefit of AZA preventing relapse (RR=0.60; 95% CI=0.37-0.95) (Khan et al. 2011). Another meta-analysis of maintenance of remission involving six studies with 124 azathioprine or mercaptopurine treated patients showed mean efficacy of 60% vs. 37% in controls (OR = 2.56; 95% CI = 1.51-4.34). However, thiopurines have not been shown to induce remission better than placebo in the same meta-analysis with four studies including 89 azathioprine or mercaptopurine treated patients showed mean efficacy of. % vs. 64% in controls (OR = 1.59; 95% CI = 0.59-4.29).

In 2021 a large analysis of the UK IBD BioResource showed thiopurine monotherapy to be effective in UC. Using 68 132 patient years of exposure, thiopurine monotherapy appeared effective (defined as no need for escalation to biologic therapy or surgery) for the duration of treatment in 2617/4968 (52.7%) patients with UC. (Stournaras et al. 2021)

Azathioprine and mercaptopurine are both hampered by nausea, hepatitis, leucopaenia and azathioprine requiring up to 30% of patients to discontinue the medication. There is some evidence that prescribing mercaptopurine after intolerance to azathioprine can be successful in up to 60% (LEES et al. 2008). Low dose azathioprine and allopurinol is much better tolerated and is tolerated in up to 75% of patients who have previously

been intolerant to either azathioprine or mercaptopurine (Smith et al. 2012; Ansari et al. 2010). Moreover, there is some evidence that patients who do not respond to azathioprine or mercaptopurine do respond to combination low dose azathioprine and mercaptopurine potentially by increasing thioguanine nucleotide levels to therapeutic range by reversing hypermethylation (Sparrow et al. 2005). Tioguanine is a more potent thiopurine which was found to be highly effective in early trials though its use is now limited due to concerns over its side effect profile especially liver injury (Dubinsky et al. 2003) and is used as a 4th line immunomodulator.

1.6.3.2 Calcineurin inhibitors

Ciclosporin introduction in the 1990s was ground breaking as it was the first drug that showed disease improvement in patients with acute severe UC who had not responded to high dose intravenous steroids for at least 7 days who would otherwise require a colectomy (Lichtiger et al. 1994). The CONSTRUCT study randomised patients to either infliximab or ciclosporin therapy as a rescue agent in steroid refractory acute severe UC and was found to have strikingly similar results with almost identical rates of colectomy and safety profile (Williams et al. 2016). However, ciclosporin performs poorly as a long-term therapy and therefore adjunctive therapy should be initiated (Harbord et al. 2017). Given the difficulty in patients having 24-hour infusions, numerous side effects and lack of long-term treatment infliximab is often preferred in many departments.

Tacrolimus is an oral calcineurin inhibitor with a similar mode of action to ciclosporin. Only 2 clinical trials have been performed in UC using tacrolimus, but both found benefit over placebo. Of note Ogata et al found 67% response rate if levels of 10-15ng/ml were achieved, and this group included a number of patients with severe disease though due to lack of power the study could not assess the effect in this group (Ogata et al. 2006).

Given that both ciclosporin and tacrolimus have poor long term response data in UC many groups have combined its use in acute severe colitis with vedolizumab which due to its slow acting nature would be a poor drug candidate in acute severe UC but can be successfully used with a calcineurin inhibitor. This is a useful alternate strategy particularly if the patient has had previous anti-TNF failure or contraindication to anti-TNF (Pellet et al. 2019).

1.6.3.3 Methotrexate

Methotrexate data is limited in UC with just 2 randomised control trials. Methotrexate was found to induce steroid free clinical remission better than placebo at week 16 (Carbonnel et al. 2016) and MERIT-UC study has demonstrated that methotrexate has no statistical difference in steroid free clinical remission at week 48 compared to

placebo (Herfarth et al. 2018). Therefore, current evidence would not support methotrexate monotherapy as maintenance therapy.

1.6.4 Biologics

There are currently 5 biologics which have been licensed and NICE approved for the treatment of UC: infliximab, adalimumab, golimumab, vedolizumab, ustekinumab. However, this list is likely to expand soon as there are a number of clinical trials in phase II or III involving novel biologics.

1.6.4.1 Anti-TNF

In the 1990s infliximab had a dramatic effect on patients with CD, which lead to a paradigm change in the treatment of severe Crohn's disease. Investigators then sought to see if infliximab would have a similar effect on UC. The seminal ACT-1 and ACT-2 trials were published in 2006. In ACT-1 364 patients entered randomisation: 69% of patients who received 5 mg/kg of infliximab and 61% of those who received 10 mg/kg had a clinical response (defined as a decrease in the Mayo score of at least 3 points and at least 30 percent, with an accompanying decrease in the sub score for rectal bleeding of at least 1 point or an absolute rectal-bleeding sub score of 0 or 1) at week 8, as compared with 37% of those who received placebo ($P<0.001$ for both comparisons with placebo). In ACT-2 364 patients were randomised: 64% of patients who received 5 mg/kg of infliximab and 69% of those who received 10 mg/kg had a clinical response at week 8, as compared with 29% of those who received placebo ($P<0.001$ for both comparisons with placebo). In both ACT-1 and ACT-2, patients who received infliximab were more likely to have a clinical response at week 30 ($P \leq 0.002$ for all

comparisons). In ACT-1, more patients who received 5 or 10 mg/kg of infliximab had a clinical response at week 54 (45% and 44%, respectively) than did those who received placebo (20%, P<0.001 for both comparisons) (Rutgeerts et al. 2005). Furthermore, colectomy rate up to week 54 was significantly reduced in both infliximab groups compared to placebo (10% v 17%, p=0.02) (Sandborn et al. 2009). ULTRA-1 and ULTRA-2 studies 390 patients were randomized to adalimumab or placebo which demonstrated significantly improved clinical remission (Mayo score ⩽ 2 with no sub score >1) with adalimumab (18.5%) over placebo (9.2%) (p=0.031) at week 8. (Reinisch et al. 2011; Reinisch et al. 2013). The PURSUIT trials demonstrated that golimumab induced steroid free clinical remission over placebo (Sandborn, Feagan, Marano, Zhang, Strauss, Johanns, Adedokun, Guzzo, Colombel, Reinisch, Gibson, Collins, Jarnerot, et al. 2014). Together these trials demonstrate a significant benefit of anti-TNF over placebo though there have been no head-to-head trials between anti-TNFs to know which may be the optimum. Moreover, with different delivery mechanisms (infliximab is intravenous whereas adalimumab and golimumab are subcutaneous) and significantly dropping costs of infliximab and adalimumab due to biosimilars has meant that access may have improved.

Immunogenicity of anti-TNFs is problematic and leads to loss of response, which can be disheartening for both patient and clinician and is particularly pertinent with infliximab. Anti-drug antibody rates vary between 7-30% of patients and are highly

likely to be influenced by the reason for taking levels (i.e. secondary loss of response much more likely than routine monitoring) as well as the rates of monotherapy (Johnston et al. 2016; Vande Casteele et al. 2015). Strategies have been devised to reduce immunogenicity including using thiopurines and methotrexate which have proven invaluable to prolong and extend the benefit of longevity of the anti-TNFs. The landmark SONIC study (Colombel et al. 2010) highlighted the benefit of using combination therapy with infliximab and azathioprine in CD and similar benefit has subsequently been shown in the UC-SUCCESS study (Panaccione et al. 2014).

Therapeutic drug monitoring has been key to maximizing the benefit of anti-TNF and personalizing therapy to the individual patient. The PANTS study coordinated out of Exeter is the largest study evaluating TDM to date. They recruited 955 patients with infliximab and 655 with adalimumab (Kennedy et al. 2019). Key messages are:

1. Primary non-response occurred in 295/1241 (23.8%) at week 14
2. Non remission occurred in 764/1211 (63.1%) at week 54
3. Nonresponse at week 54 was associated with low drug concentrations at week 14 (infliximab: odds ratio 0·35 [95% CI 0·20-0·62], p=0·00038; adalimumab: 0·13 [0·06-0·28], p<0·0001)
4. Optimal week 14 drug concentrations associated with remission at both week 14 and week 54 were 7 mg/L for infliximab and 12 mg/L for adalimumab

5. Combination immunomodulator (thiopurine or methotrexate) therapy mitigated the risk of developing anti-drug antibodies (hazard ratio 0·39 [95% CI 0·32-0·46] for infliximab; 0·44 [0·31-0·64] for adalimumab; p<0·0001 for both).
6. Immunomodulator use was associated with reduced non-response at week 54 (odds ratio 0·56 [95% CI 0·38-0·83], p=0·004) but this was not shown with adalimumab

1.6.4.2 Anti-integrins

Vedolizumab is the only currently licensed anti-integrin therapy. The GEMINI study was a double-blind randomised control trial which took 895 patients across 2 cohorts and randomised to vedolizumab or placebo. The study showed statistically significant clinical response (defined as a reduction in the Mayo Clinic score of at least 3 points and a decrease of at least 30% from the baseline score, with a decrease of at least 1 point on the rectal bleeding subscale or an absolute rectal bleeding score of 0 or 1) at week 6 of vedolizumab (47.1%) over placebo (25.5%) p<0.001. At week 52, 41.8% of patients who continued to receive vedolizumab every 8 weeks and 44.8% of patients who continued to receive vedolizumab every 4 weeks were in clinical remission (Mayo Clinic score ⩽2 and no sub score >1), as compared with 15.9% of patients who switched to placebo p<0.001 (Feagan et al. 2013). More recently in the VARSITY trial: a head to head trial of vedolizumab and adalimumab, has shown vedolizumab to have improved clinical remission and endoscopic improvement at week 52 (Sands, Peyrin-

Biroulet, et al. 2019). However, this study is not without criticism due to potential issues with patient selection with 20% of patients having documented prior anti-TNF failure, lack of TDM and no ability for drug escalation of either medication.

Vedolizumab has been shown to be beneficial in many patients who have failed an anti-TNF previously (Feagan et al. 2017) providing a useful alternative medication in those patients who have already failed anti-TNF. Furthermore, given its mode of action of blockade of ingress of lymphocytes into the gastrointestinal system vedolizumab presents itself as a particularly useful agent to be used in patients with compromised immune systems (e.g., bone marrow transplantation, chemotherapy), infective complications (e.g., bronchiectasis, or tuberculosis) and oncological disease where there may be theoretical interactions with cytokine altering biologics.

1.6.4.3 Ustekinumab

Ustekinumab is an anti-IL12p40 drug which acts against both the IL-12 and IL-23 receptors. The UNIFI study published in 2019 randomised 961 patients with UC to ustekinumab or placebo in a double blind, randomised study design. At week 8 ustekinumab had statistically higher rates of clinical remission (defined as a total score of $\leqslant 2$ with no sub score >1) of 15.6% versus placebo (5.3% with $p<0.001$. In those who responded at week 8 they were re-randomised to either 12 weekly or 8 weekly

ustekinumab or placebo. At week 44 statistically higher rates of clinical remission were seen in the ustekinumab groups (38.4% for 12 weekly, 43.8% for 8 weekly) versus placebo (24%) P0.002 and p<0.001. Ustekinumab showed statistically significant achievement of important secondary endpoints such as corticosteroid free remission, endoscopic improvement at week 8 and 44. (Sands, Sandborn, et al. 2019).

1.6.4.4. Mirikizumab

Mirikizumab is an antibody directed against IL23p19 subunit of the IL-23 receptor. LUCENT-1 was the induction trial mirikizumab (300mg at weeks 0, 4 and 8) to placebo which randomised 1281 patients. At week 12 higher rates of clinical remission were seen with mirikizumab versus placebo (24.2% vs. 13.3%, P<0.001). LUCENT-2 was the maintenance trial which took 544 patients who had clinical response in LUCENT-1 (defined as a decrease of ⩾2 points in the modified Mayo score, with a decrease of ⩾30% from baseline, plus either a decrease from baseline of ⩾1 point in the rectal bleeding subscore or a rectal bleeding subscore of 0 or 1) with mirikizumab and re-randomised them to mirikizumab 200mg sc or placebo (mirikizumab withdrawal). At week 40 mirikizumab treated patients had a higher clinical remission than placebo (mirikizumab withdrawal) patients (49.9% vs. 25.1%, P<0.001) (D'Haens et al. 2023).

It has been approved by NICE and is in use in clinical practice.

1.6.4.5. Risankizumab

Risankizumab is also an antibody directed against IL23p19 which has been used widely for the treatment of psoriasis and psoriatic arthritis. The INSPIRE study recruited eligible patients (18-80 years) with moderately to severely active ulcerative colitis (Adapted Mayo score of 5 – 9 points) and an endoscopic subscore of 2-3 (per central review) were enrolled. Patients were required to have demonstrated intolerance or inadequate response to conventional and/or advanced therapies (biologics, JAK inhibitors, and S1P receptor modulators). 975 patients were randomized 2:1 to receive either risankizumab 1200 mg intravenously or placebo at weeks 0, 4, and 8. The primary endpoint was clinical remission (per Adapted Mayo Score defined as stool frequency subscore $\leqslant$ 1 and not greater than baseline, rectal bleeding subscore of 0, and endoscopic subscore $\leqslant$ 1 without friability) at Week 12. A significantly greater proportion of patients receiving Risankizumab v placebo achieved clinical remission (20.3% vs. 6.2%, adjusted treatment difference 14.0% [95% CI, 10.0%-18.0%], p < .00001). (Loftus Jr 2023)

Patients who achieved a clinical response in the INSPIRE induction studies were randomized 1:1:1 in the COMMAND study to risankizumab 180 mg, 360 mg, or placebo (risankizumab withdrawal) subcutaneous (SC) treatment every 8 weeks for 52

weeks. The primary endpoint was clinical remission at week 52. Patients receiving Risankizumab 180mg (40.2%) and 360mg (37.6%) achieved significantly higher rates of clinical remission vs placebo (risankizumab withdrawal) (25.1%) (adjusted treatment difference vs placebo: 16.3% and 14.2%, respectively; $P \leqslant .01$) (Louis et al.)

The above studies have only been released as abstracts at this time. Full publication is expected in the near future and NICE assessments are underway to establish the positioning that risankizumab may have in the treatment of ulcerative colitis.

1.6.5 Small molecules

A new class of drugs has emerged which have been broadly labelled descriptively as 'small molecules' and are not biologics as they are not derived from living organisms or their by-products. There are a number of differences compared to biologics: their structure is small (i.e., < 1000 Da) compared to biologics which are large (infliximab is 140 kDa), their action is intracellular, they are administered orally, do not induce immunogenicity nor antibody formation.

1.6.5.1 JAK inhibitors

1.6.5.1.1 Tofacitinib

In the OCTAVE Induction 1 trial, clinical remission (a total Mayo score of ⩽2, with no sub score >1 and a rectal bleeding sub score of 0) at 8 weeks occurred in 18.5% of the patients in the tofacitinib group versus 8.2% in the placebo group (P=0.007); in the OCTAVE Induction 2 trial, remission occurred in 16.6% versus 3.6% (P<0.001). In the OCTAVE Sustain maintenance trial patients who had clinical response were re-randomised to placebo, tofacitinib 5mg BD or tofacitinib 10mg BD, remission at 52 weeks occurred in 34.3% of the patients in the 5-mg tofacitinib group and 40.6% in the 10-mg tofacitinib group versus 11.1% in the placebo group (P<0.001 for both comparisons with placebo) (Sandborn et al. 2017).

Response rates are quick with the studies show separation between drug and placebo as early as day 3 (Hanauer et al. 2019) meaning that bridging corticosteroid is unlikely to be required. Furthermore, response rates are similar for biologic naïve and biologic experienced patients, which contrasts with trials with biologics.

1.6.5.1.2 Filgotinib

Filgotinib, a specific JAK-1 inhibitor, was investigated in the SELECTION trial involving 1348 patients in a multicentre international study. This showed filgotinib induced clinical remission at week 10 statistically significantly more than placebo with 200mg dose (induction study A 26·1% vs 15·3%, difference 10·8%; 95% CI 2·1–19·5, p=0·0157; induction study B 11·5% vs 4·2%, 7·2%; 1·6–12·8, p=0·0103). At week 58, 37·2% of patients given filgotinib 200 mg had clinical remission versus 11·2% in the respective placebo group (difference 26·0%, 95% CI 16·0–35·9; p<0·0001). but not with 100mg dose (clinical remission was not significantly different between filgotinib 100 mg and placebo at week 10, but was significant by week 58 (23·8% vs 13·5%, 10·4%; 0·0–20·7, p=0·0420). (Feagan et al. 2021). Filgotinib is now NICE approved and used in clinical practice.

1.6.5.1.3 Upadacitinib

Upadacitinib is a selective JAK-1 inhibitor investigated in 2 phase III trials U-ACHIEVE and U-ACCOMPLISH including a total of 996 patients. In U-ACHIEVE, a significantly higher proportion of patients receiving upadacitinib (26.1%) vs placebo (4.8%) achieved clinical remission at week 8 (adjusted treatment difference [95% CI], 21.6% [15.8, 27.4]; P<0.001 (Danese et al. 2021). In U-ACCOMPLISH, a significantly higher proportion of patients receiving upadacitinib (33.5%) versus placebo (4.1%) achieved clinical remission at week 8 (adjusted treatment difference: 29.0% [23.2, 34.7]; P<0.001) (Vermeire et al. 2021). Furthermore, upadacitinib appears to have a very prompt clinical effect with patients treated with upadacitinib experienced significant

improvement in daily symptoms, with significantly more subjects achieving stool frequency score ⩽1 ($p<0.001$), rectal bleeding score of 0 ($p<0.05$), as early as day 1 and maintained through day 14. A significantly higher percentage of patients who received UPA 45 mg OD compared to PBO, achieved abdominal pain=0 and the absence of bowel urgency within 3 days of beginning treatment through day 14 ($p<0.05$) (Vermeire et al. 2022). Upadacitinib is an exciting drug given is prompt clinical effect and large delta from placebo and is in clinical practice.

1.6.5.2 Sphingosine-1-phosphate receptor modulators

Ozanimod is an oral S1P modulator which has been investigated in ulcerative colitis in the TRUENORTH study. In the induction period, 645 patients were included in cohort 1 and 367 in cohort 2; a total of 457 patients were included in the maintenance period. The incidence of clinical remission was significantly higher among patients who received ozanimod than among those who received placebo during both induction (18.4% vs. 6.0%, $P<0.001$) and maintenance (37.0% vs. 18.5% [among patients with a response at week 10], $P<0.001$). It is now available for use in clinical practice.

1.7 Choice of treatment

Current treatment guidelines including those of ECCO endorse inducing remission in UC with aminosalicylates +/- corticosteroids then if there is relapse or inadequate

response to commence a thiopurine (Harbord et al. 2017; Lamb et al. 2019). If there is inadequate response then commence either: infliximab, adalimumab, golimumab, vedolizumab, ustekinumab or tofacitinib. Up until recently there was no direct comparison available to compare each of these medications and therefore choice was dependent on clinician preference, patient preference, past medical history, and cost. Different medications may suit one patient over another e.g., intravenous infusion may be inconvenient so chose adalimumab or golimumab, needle phobia may choose tofacitinib, chronic infection choose vedolizumab.

The first head-to-head study biologic study in UC was published in 2019. The VARSITY study compared the efficacy of vedolizumab and adalimumab in UC and showed better clinical remission and better endoscopic remission at week 54 (Schreiber et al. 2019). This study for the first time has raised the question of whether using vedolizumab before using anti-TNF would benefit more patients. However, given that the cost of vedolizumab is higher than that of biosimilar anti-TNF and that it requires intravenous infusions some patients and commissioning groups may still favour anti-TNF as first line therapy. This is a constantly evolving area, and more head-to-head studies are currently recruiting to answer some of these important questions.

Recently a network meta-analysis using trial data has shown that upadacitinib may be the most effective therapy for treating UC in a multitude of clinical, endoscopic and histological endpoints (Burr et al. 2021).

Regardless of which treatment is chosen it is important to assess a patient objectively both before treatment as well as after a period of time, most commonly 14-16 weeks after induction. There are many ways to objectively assess disease including faecal calprotectin, CRP, radiologically and endoscopically. UC by its nature of being a distal colonic disease allows easy endoscopic assessment of disease, which is the gold standard. However given the constraints on endoscopy due to ever growing demand this proves to be a challenge in many hospitals and therefore the surrogates of calprotectin, intestinal ultrasound (Maaser et al. 2019), clinical symptoms and CRP which correlate well with endoscopic findings may be used instead (Reinisch et al. 2018; Kawashima et al. 2016).

As highlighted by the PANTS study therapeutic drug monitoring can be helpful to identify patients who may benefit from escalated drug dosing particularly if they have had a suboptimal response (Kennedy et al. 2019).

1.8 Future treatments

IBD is undergoing a paradigm shift in the expanding number and mechanism of action of treatments, which is creating genuine excitement in IBD physicians and patients. In 2023 there have been 4 new drugs approved for use in UC (mirikizumab, upadacitinib, filgotinib and ozanimod) expanding the number of advanced therapies to 10. Risankizumab is expected to be available in 2024.

Whilst it is never applicable to directly compare efficacy of medications across diseases, a review of the psoriasis IL-23 inhibition literature provides interesting conclusions. In Crohn's disease the SEQUENCE study compared risankizumab v ustekinumab in patients who had previously failed or been intolerant to anti-TNF. It has shown statistically improved endoscopic remission at week 48 with risankizumab v ustekinumab (31.8% v 16.2%, p< 0.0001) (Peyrin-Biroulet et al.). Furthermore, in psoriasis, anti-IL-23p19 therapy is more effective than anti-IL12p40 therapy in psoriasis with 90% or greater reduction in the Psoriasis Area and Severity Index (PASI) score of 77% (64 of 83 patients) for risankizumab (90-mg and 180-mg groups pooled), as compared with 40% (16 of 40 patients) for ustekinumab (P<0.001) (Papp et al. 2017). If comparable results are observed in IBD, selective IL-23 blockade could be a 'game changer' in the field of IBD treatments.

Combination biologics have been investigated in the VEGA study: 214 anti-TNF naïve patients with UC were randomised to guselkumab, golimumab or combination guselkumab + golimumab. The primary endpoint of clinical remission (modified Mayo score: stool frequency 0 or 1, rectal bleeding score of 0 and endoscopy score of 0 or 1) at week 12 was 36.6% in combination group, 22.2% in golimumab alone (p=0.058) and 22.1% in guselkumab alone (p=0.041). Whether this combination will be clinically useful is unclear given the less than impressive results, but the concept of combination therapies is likely to expand with time and may prove useful to induce remission without the need for steroids (e.g., anti-TNF or JAK inhibition) and maintain long-term remission with a safe drug (e.g., vedolizumab)

1.9 Predicting response to therapies in IBD

There is now an exciting opportunity to exploit our expanding knowledge of IBD immunopathogenesis to better ascertain which immune mechanisms of disease exist in individual patients with a view to tailoring the use of specific therapeutic interventions. In the era of sensitive experimental platforms and big data analysis tools the possibility of transitioning this promise into a reality grows ever closer. In this thesis I will consider how a molecular understanding of IBD could be harnessed to build reproducible laboratory assays to predict drug response in individual patients to deliver personalised IBD care. This section will not consider general factors associated with drug

responsiveness such as demographic features, clinical phenotype, or drug exposure parameters (essentially related to therapeutic drug monitoring and pharmacokinetic/pharmacodynamic factors), since these aspects have been extensively reviewed elsewhere (Ding, Hart, and De Cruz 2016; Kopylov and Seidman 2016).

Despite the success of anti-TNF therapy in IBD, only subgroups of patients have benefitted. Depending on the endpoints chosen, between 40-60% of patients respond to anti-TNF treatment (Hanauer et al. 2002; Colombel et al. 2007). Broadly similar efficacy is observed with other approved biologics such as vedolizumab (Sandborn et al. 2013; Feagan et al. 2013) and ustekinumab (Feagan et al. 2016). Currently, predictive tools are mostly confined to phenotypic, biochemical, and demographic variables that lack specificity or reproducibility. Most data reported has focussed on anti-TNF therapy and typically comprises retrospectively defined predictors with small patient numbers in each study. Data are occasionally conflicting and has seldom been validated in independent cohorts. Such reports are prone to publication bias since features that do not predict response are less likely to be published. Of course, reliable prediction of response to biologics would enable initiation of the most effective therapy, facilitating time-efficient control of disease and would limit disease complications including irreversible structural damage. This strategy would additionally improve the cost effectiveness of medical therapy by avoiding ineffective therapies and potentially increasing the reach of expensive biological therapies to patient groups

previously denied access by resource restricted healthcare systems. Techniques informed by an understanding of the immune mechanisms of disease in individual patients promise to uncover new biomarker platforms as tools for precision medicine in IBD.

1.9.1 Transcriptomics

Transcriptomics, the study of the whole expression and function of RNA in a cell or sample, has become an integral tool for analysis of health and disease. In the past decade, due to falling costs of sequencing technologies, increased analytical expertise and novel computational tools to allow large scale data analysis. RNA sequencing (RNA-seq) uses high throughput analysis of the entire transcriptome (all mRNA transcripts present in a particular sample). RNA-seq has supplanted gene expression microarray studies, which use predetermined sets of probes to quantify defined mRNAs. Understanding which genes are being transcribed in diseased tissue, individual immune cells, or indeed in peripheral blood affords quantitative and qualitative insights into which immune pathways might be activated or repressed in an individual patient and how these changes compare across patient populations.

There are multiple challenges associated with the use of transcriptomics particularly when attempting the development of a biomarker. RNA expression is governed by multiple of parameters, regulators and represents a single time point, in that sample, in

an individual patient. This 'snapshot' provides a huge amount of potential insight in the status of the intracellular workings of the tissue however there are limitations with this approach. Inflammation in IBD is not uniform in terms of position in the colon as well as temporally. Therefore, transcriptomic insights are limited as by the time that the data has been analysed (even if a few short days) the transcriptomic pattern will have changed, potentially significantly, such that drug decisions which were based on the original transcriptomic pattern may now be invalid. Furthermore, biopsies from the sigmoid colon may not be representative of the inflammation in the rest of the colon even if a few millimetres away.

RNAseq has been used surprisingly infrequently with only a handful of published studies. Taman *et al* (Taman et al. 2018) published a series of 14 newly diagnosed UC patients and compared the gene expression to that of 16 healthy controls. They found 1480 differentially expressed genes with $P<0.05$ and FDR >2 including many chemokine receptors and ligands, TNF receptor family, ICAM-1, NOD2 and STAT3. They compared their DEG list to GWAS identified IBD susceptibility genes and found significant cross over with 71 upregulated (24%) and 8 downregulated (2.7%) transcripts. Interestingly they found significant differences in the results dependent on the sex of the patient with 8 transcripts identified as differentially expressed between the sexes. This small study provides some interesting insights into transcriptomic patterns in UC,

but validated cohort studies would be required to establish if the sex differences were corroborated.

Haberman *et al* (Haberman et al. 2019) have published the largest case series of RNAseq in UC by comparing differentially expressed genes between 206 newly diagnosed, treatment naive paediatric UC samples and 20 healthy controls. They found 5296 genes differentially expressed (FDR < 0.001 and fold change (FC) ⩾ 1.5) in comparison to controls. Functional annotation analysis demonstrated showed highest enrichment for increased lymphocyte activation and associated cytokine signalling, and a robust decrease in mitochondrion, aerobic tricarboxylic acid (TCA) cycle and metabolic functions an observation which had not been previously seen and was ratified by functional experiments. Furthermore, they identified a transcriptomic signature associated with corticosteroid response at week 4, which was significantly enriched in patients with a poorer outcome (i.e., no clinical response to corticosteroids, anti-TNF or anti-integrins or calprotectin >250µg/g).

Other studies have utilised colonic tissue transcriptomics to predict treatment response in IBD. The first published study by Arijs and colleagues used a mRNA microarray platform to profile gene expression in colonic biopsies from UC patients from the ACT1 trial (Rutgeerts et al. 2005), and a separate cohort from Leuven before the initiation of infliximab therapy (Arijs, Li, et al. 2009). Response was defined as endoscopic remission

(Mayo score 0 or 1) and histological remission (Geboes score 0 or 1) at week 4 or 6, depending on whether infliximab was given as a single dose or loading regime. They demonstrated 212 transcripts that were differentially expressed in baseline biopsies (i.e., immediately prior to implementation of infliximab) in patients who would subsequently respond to infliximab versus those who would not. Notably, the five most differentially expressed genes (*TNFRSF11B, STC1, PTGS2, IL13RA2, IL11*) predicted endoscopic remission to infliximab with 95% sensitivity and 85% specificity (Arijs, Li, et al. 2009).

By analysing mRNA expression of different cytokines in mucosal biopsies, West *et al* identified a newly implicated cytokine oncostatin M (*OSM*) and its receptor (*OSMR*), and a co-expressed transcriptional module (i.e. transcripts whose expression patterned followed *OSM* and *OSMR*) in mucosal biopsies taken prior to institution of anti-TNFα to predict non-response to anti-TNF in UC and CD (West et al. 2017). Interestingly, functional studies confirmed a pathogenic role for OSM in pre-clinical models of IBD that are resistant to anti-TNF treatment. Reposited transcriptional data of intestinal biopsies from the ACT1 trial (Rutgeerts et al. 2005) demonstrated high expression of transcriptional modules that were co-expressed with OSM to be associated with non-response to infliximab therapy with a high area under the curve of 0.99 with OSM and 0.83 with OSMR. These results suggests that anti-TNFα non-response is driven by an alternative, non-TNFα, driven pathways which confers poor response to anti-TNFα

therapy. These data again highlight the close relationship between understanding mucosal immunopathology in IBD and prediction of drug response.

Deconvolution techniques can provide invaluable insights not heralded by whole genome analysis due to multiple variations of gene expression by different cell types effectively clouding each other. Whole genome expression datasets are analysed by estimating the immune cell contribution to the gene expression so producing estimations of gene expression by cell type. Gaujoux *et al* analysed publicly accessible datasets from landmark IBD clinical trials and found that high plasma cell and macrophage abundance were associated with non-response to anti-TNF therapy. In an independent cohort they examined plasma cell frequencies histologically and found that high plasma cell frequency associated with non-response to anti-TNF therapy (AUC 74%, p=0.002). Furthermore, the group analysed differentially expressed genes between anti-TNF responders and non-responders having adjusted for abundance of plasma cells and macrophages. They found the CCL7-CCR2 network was significantly enriched, and TREM-1 was identified as an upstream regulator in anti-TNF non-responders. Using a prospective cohort of CD patients prior to commencing anti-TNF therapy they analysed blood expression of these TREM-1/CCL7/CCR2 genes and found significantly downregulated TREM-1 in non-responders compared to responders (p-0.007, AUC=0.94) (Gaujoux et al. 2019).

However, a study by Verstock *et al* (Verstockt et al. 2019) found opposing results. They found that TREM1 expression in blood of 30 patients with UC and 24 patients with CD before commencing anti-TNF therapy was downregulated in anti-TNF responders (UC, $P = 0.01$; CD, $P = 0.07$). The difference in results of these two studies could not be starker: one found that TREM1 was downregulated in anti-TNF responders, and the other found that TREM1 was downregulated in anti-TNF non-responders. The reason might, in part, come from the methods used, as Gaujoux *et al*. defined response using clinical scores whereas Verstock *et al.* used endoscopic scores, which are likely to better reflect true disease activity and therefore might provide more robust outcome data. Given the opposing data from a different group, further independent cohort studies are required to clarify whether TREM1 could be utilized as a biomarker prior to commencing anti- TNF therapy in patients with IBD. If confirmed, it would enable accessible and simple testing with quantitative PCR (qPCR) prior to commencing anti-TNF therapy. This exciting study utilising innovative technologies and highly sophisticated analysis techniques has identified an important potential biomarker, which has previously been unrecognised and may allow accessible and relatively simple testing with qPCR prior to anti-TNF commencement.

Tissue transcriptomics have also been exploited to predict response to anti-integrin therapy. The Leuven group published prospectively took colonic biopsies and performed RNAseq on 31 patients commencing vedolizumab for UC and CD. They

demonstrated differential expression of 44 genes (P<0.005 and FDR<0.25). Further analysis showed that when they analysed expression of 4 genes (RGS13, DCHS2, MAATS1, and PIWIL1) they could predict response with an accuracy of 80% which was validated in independent cohorts (Verstockt et al. 2020).

A phase II trial in UC evaluating the safety and efficacy of etrolizumab, a mAb targeting the β7 integrin subunit, has shown that the expression of transcripts encoding integrin αE and granzyme A were significantly higher in baseline biopsies of patients who responded to etrolizumab in comparison with those who did not. A higher proportion of those with high levels of *GZMA* mRNA (41%) or *ITGAE* mRNA (38%) than those with low levels of *GZMA* (6%) or *ITGAE* mRNA (13%) achieved clinical remission (Tew et al. 2016).

1.9.2 Molecular endoscopy

Recently, a novel approach that is directly derived from insights into the molecular mechanisms of disease has been evaluated to predict therapeutic efficacy of anti-TNF treatment in CD. In vivo topical application of a fluorescent labelled anti-TNF antibody directed onto the diseased mucosa in conjunction with endoscopic confocal laser endomicroscopy (CLE), enabled visualisation of mTNF bearing mucosal cells in vivo. The proportion of $mTNF^{+}$ bearing cells in the mucosa can be readily quantified (see Figure

1.8.1 for schematic of molecular endoscopy process). Patients with high numbers of mTNF^{+} cells showed significantly higher clinical response rates at week 12 (92%) upon subsequent anti-TNF therapy as compared to patients with low numbers of tissue mTNF^{+} cells (15%) (Atreya et al. 2014). Furthermore, clinical response was sustained over a follow-up period of one year and associated with mucosal healing (Atreya et al. 2014).

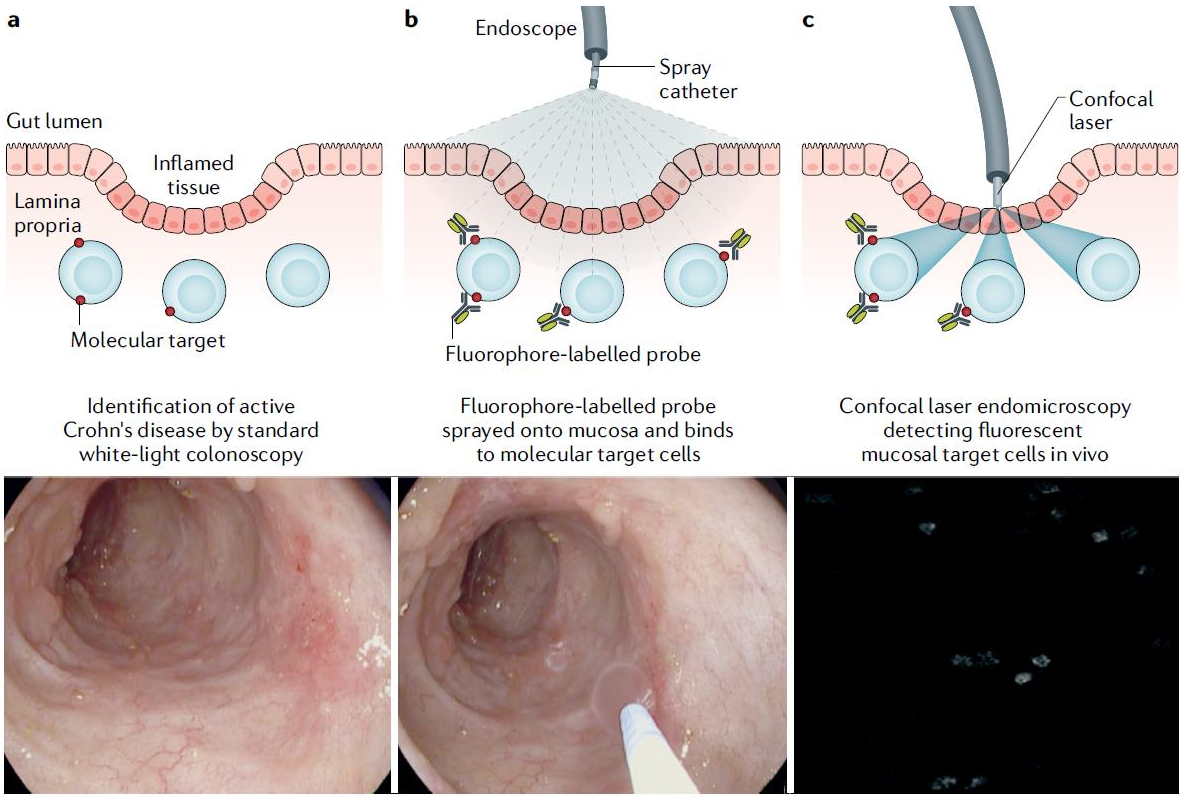


Figure 1.9.1 schematic of principles of confocal laser endomicroscopy showing endoscopic images and cartoon representation of mucosal molecular targets. (1) active CD identification by endoscopy, (2) fluorescent labelled anti-TNF sprayed onto the mucosa using a spray catheter (bottom right of picture) and confocal laser projected onto fluorescent labelled mucosa (3) confocal laser

endomicroscopy image of fluorescent labelled molecular target cells which can be quantified (Digby-Bell et al. 2020)

A subsequent pilot study has demonstrated a correlation between the therapeutic efficacy of vedolizumab in CD and the number of α4β7 expressing cells in the mucosa after applying fluorescent labelled vedolizumab onto intestinal biopsies ex vivo, followed by CLE (Rath et al. 2017). However, this was only demonstrated in 5 patients, so a larger cohort is required.

These findings suggest that molecular endoscopic imaging with fluorescent labeled antibodies directed against specific immune molecules could be exploited to predict responsiveness to subsequent biological therapy and may thus provide a rational approach for personalized medicine. However, molecular endoscopy requires validation in multi-center studies before being adopted into clinical practice.

1.9.3 Germline genetics

Although GWAS has been productive in the identification of susceptibility loci in IBD (currently around 240) (Jostins et al. 2012; Liu et al. 2015; de Lange et al. 2017), analysis of germline genetic variation has been disappointing as a clinically useful tool for predicting response to treatment. The most well-known susceptibility gene, *NOD2*, confers a relative risk of CD of 3.0 in heterozygotes and 23.4 in homozygotes (Cuthbert et al. 2002). However, two well designed studies have failed to show an association between *NOD2* genotype and response to infliximab (Mascheretti, Hampe, Croucher, et al. 2002; Vermeire et al. 2002). Likewise, polymorphisms of the TNFα receptor, does

not to differentiate infliximab responders and non-responders (Mascheretti, Hampe, Kuhbacher, et al. 2002; Pierik et al. 2004). IL-23 receptor (*IL-23R*) polymorphisms are also strongly associated with IBD (Jostins et al. 2012; Liu et al. 2015; de Lange et al. 2017), and interestingly, homozygosity for 'high risk' *IL-23R* variants was associated with improved response rates to infliximab in comparison with patients who were homozygous for 'low risk' *IL-23R* variants (74.1 vs. 34.6%) (Jurgens et al. 2010).

Given the central role of TNF in regulating apoptosis (Atreya et al. 2011), genetic variation at loci encoding genes involved in apoptosis have also been screened as potential biomarkers of anti-TNF responsiveness. In a cohort of 287 patients, Fas ligand-843 CC/CT was predictive of response to infliximab in luminal and fistulising disease CD compared to the TT genotype (75% v 38%) (Hlavaty et al. 2005). In addition, patients with luminal CD with caspase-9 93 TT genotype (n = 9) all responded to infliximab (100%) versus 67% (n = 147) with the CC and CT genotype (Hlavaty et al. 2005).

A number of other loci have also been reported. A Spanish study found that homozygous variants of the IBD5 locus conferred a relative risk of non-response to infliximab of 3.9 in CD, but not in UC (Urcelay et al. 2005). Polymorphisms at the *FCGR3A* locus, encoding IgG Fc receptor IIIa, was associated with response to infliximab in CD (Louis et al. 2004), but failed to replicate in a larger cohort (Louis et al. 2006).

Although monogenetic disorders are very rare in IBD overall, they are enriched in the paediatric population with an increasing proportion of patients affected the earlier the onset of disease, and the highest prevalence found in those aged less than 6 years - a group known as very early onset IBD (VEO-IBD)(Muise, Snapper, and Kugathasan 2012; Uhlig et al. 2014). Nevertheless, knowledge of the underlying genetic defect is helpful in informing personalized treatment strategies. For example, mutations in *IL10RA* and *IL10RB* have a much better outcome with Haematopoietic Stem Cell Transplantation (HSCT) (Glocker et al. 2009; Kotlarz et al. 2012) than patients with epithelial defects such as EPCAM and TCC7A (Kammermeier et al. 2016), which respond less well and therefore alternative strategies may be implemented. *NLRC4* mutations lead to excessive, life threatening inflammation associated with increased inflammasome activity and excessive IL18 production, which was successfully treated by IL18 blockade (Canna et al. 2017). These data also highlight the potential importance of interrogating host phenotype (in this case immunophenotype) in conjunction with genetic data. Patients with *LRBA* mutations, which results in loss of the immunomodulatory molecule CTLA4 on Tregs and effector T-cells, responded well to abatacept, a CTLA4 (cytotoxic T-lymphocyte antigen-4) fusion drug(Lo et al. 2015). However, selection of patients on whom to perform whole genomic sequencing on has yet to be defined though young age of onset is likely to be the most informative marker (Uhlig et al. 2014).

In summary, genetic studies have yielded some promising findings regarding predicting response to therapies, but like other platforms they await validation in larger cohort studies. Although rare overall, characterising genetic defects in patients with VEOIBD, which is enriched for monogenetic disorders, can be immensely helpful in informing personalised therapeutic approaches – especially when combined with immune response profiling.

1.9.4 Immunoprofiling

Immune response pathways can be profiled and quantified in a variety of other ways including flow cytometry (identifying the phenotype of individual immune cells) and proteomics (measuring cytokines, chemokines, or other immune proteins) in the gut, blood or biofluids from IBD patients.

Schmidtt *et al* showed that in patients with Crohn's disease treated with anti-TNF that responders (where response was defined as the subsequent colonoscopy after initiation of adalimumab or infliximab showed SES-CD <5) had a statistically significant increase in TNFR2 expression in intestinal biopsies prior to commencing anti-TNF. Furthermore, they showed that during treatment a statistically increased number of TNFR2+IL23R+ T cells in the mucosa of anti-TNF resistant patients with Crohn's disease compared with treatment naïve or anti-TNF responders (Schmitt et al. 2017). This study indicates that alterative immune pathways may only be activated under certain conditions or

immunological pressures such as anti-TNF therapy. It also reveals the crucial role that IL-23 plays in the resistance of anti-TNF in Crohn's disease by allowing escape of apoptosis via a STAT3 mediated mechanism. However, as the TNFR2+IL23R+ T cells are not elevated prior to commencing anti-TNF this proves to be less useful in clinical practice except to show that anti-IL23 therapies may be useful as a second line therapy.

Baseline concentration of IL-22 in serum of CD patients has been shown to predict response to anti-IL-23p19 therapy (Sands et al. 2017). In this phase IIa trial, more than 60% of CD patients with elevated levels of serum IL-22 (>15.6 pg/ml) at baseline achieved a clinical response (CD activity index (CDAI) fall >100 points) at week 8 following 2 doses of brazikumab (given at week 0 and week 4), in comparison to placebo treated patients, or brazikumab treated patients with serum IL-22 <15.6pg/mL, where clinical response rates were comparable at about 30%. Strikingly, the use of immune profiling stratification in this cohort achieved impressive response rates of brazikumab, with a delta >30% over placebo, or "biomarker negative" brazikumab treated patients (Sands et al. 2017). Importantly, IL-23 is the canonical cytokine responsible for triggering IL-22 production(Chen et al. 2016; Geremia, Arancibia-Carcamo, et al. 2011), so it is conceptually attractive to envisage that patients with the highest levels of IL-22, are those most likely to have upstream IL-23 activation. Although these results, if replicated, could unveil anti-IL-23p19 therapy as a game changing biologic when stratified by serum IL-22 concentrations as a companion

diagnostic, caution should be taken when interpreting these results. Response was defined as a fall in CDAI, a score highly weighted to a patient symptom reported outcome which may not reflect the true activity of CD when compared with endoscopic findings(Jones et al. 2008; Regueiro et al. 2011). Furthermore, the IL-22 biomarker was defined retrospectively, so larger validating cohort studies are required before this approach could be adopted. Serum cytokine measurement would represent a cheap and technically simple biomarker, which could be widely adopted.

1.9.5 Microbiome

The complex interactions between host immunity and the microbiome are slowly being unravelled, and whilst strictly speaking the microbiome is not 'host' immunity, the microbiome has a major impact shaping host immune responses, especially in the gut (Manichanh et al. 2012; Round and Mazmanian 2009; Littman and Pamer 2011) The dysbiosis present in IBD includes reduced diversity (Sokol et al. 2008; Ott et al. 2004) (a consistent observation) and expansion/contraction of certain phyla/species (inconsistently reported) (Kostic, Xavier, and Gevers 2014). In agreement with these observations, correction of the dysbiosis in UC with faecal microbiota transplantation, induces clinical and endoscopic responses (Paramsothy et al. 2017; Moayyedi et al. 2015). Ananthakrishnan and colleagues analysed the faecal microbiota in a cohort of CD and UC patients commencing vedolizumab. CD patients with higher α-diversity had a higher rate of remission following treatment with vedolizumab at week 14.

Responders also had a higher abundance of both *Roseburia inulinivorans* and *Burkholderiales* species. Statistically significant differences were not found in UC (Ananthakrishnan et al. 2017).

1.9.6. Epigenetics

DNA methylation profiling has been shown to be differentially expressed by different phenotypes in Crohn's disease. To evaluate whether DNA methylation profiles could act as a biomarker to predict response to IBD therapies Joustra *et al* performed a study on 184 adult CD patients prior to and after a median of 28 weeks following biological treatment with adalimumab, vedolizumab or ustekinumab in a discovery (n=88) and independently collected internal validation cohort (n=96) using the Illumina EPIC BeadChip. Patients were comprehensively evaluated for response by a combination of endoscopic response (⩾50% reduction in SES-CD score) and steroid-free clinical response (⩾3 point drop in HBI or HBI ⩽4 AND no systemic steroids) and/or biochemical response (⩾50% reduction in C-reactive protein (CRP) and faecal calprotectin or a CRP ⩽5 g/mL and faecal calprotectin ⩽250 µg/g). 58 adalimumab treated patients (responder=29, non-responders=29), 64 vedolizumab treated patients (responders=36, non-responders=28) and 62 ustekinumab treated patients (responders=30, non-responders=32) were included. Prior to treatment distinct panels of 100 adalimumab, 22 vedolizumab and 68 ustekinumab associated loci were identified that, in combination, predict clinical and endoscopic response with high

accuracy (AUC adalimumab=0.73, vedolizumab=0.89 and ustekinumab=0.94) upon validation. This well designed study using robust response definitions has the potential of being a highly valuable tool to aid prediction of response to IBD therapies.

1.10. Challenges of personalised medicine in IBD

Identifying a suitable cohort is crucial: if all patients with IBD are included then this will include various stages of disease including exposure to multiple drugs and surgery. Hence, inception cohorts are particularly attractive but also challenging to identify prior to their diagnostic colonoscopy. Furthermore, pre-selecting patients in which the biomarker may be enriched such as very early IBD, or paediatric patients is also an attractive option. However, the cohort should also be representative of the wider population which is being targeted so should include patients of the relevant age, sex and race (Denson et al. 2019). These highly valid requirements require significant planning and time to identify a sufficient quantity and quality of samples.

Identifying a suitable endpoint dependent upon the aim of the biomarker and the cohort examined. For example, SES-CD scoring for perianal or isolated small bowel Crohn's disease may not be representative. Endpoints across studies are heterogenous and hence not possible to directly compare. Therefore, objective, validated, defined and relevant scoring systems would provide a platform to permit biomarkers to be

compared. (Alsoud, Vermeire, and Verstockt 2022) This would require significant coordination of commercial studies which would rest of the governing bodies i.e., FDA or EMA to dictate the endpoints and inclusion / exclusion criteria to try to provide comparison across studies.

There are also platform specific challenges. The field of transcriptomics would be challenging to adopt into clinical practice due to the expense of the technology, paucity of bioinformatic support and analysis expertise, and a long turnaround time from biopsy to result which may be too long to wait for a clinically urgent patient group. However, the costs of genome-wide transcriptomic platforms including RNA-seq continues to fall (currently available for as low as $200 per sample), and comprehensive gene expression profiling may provide information beyond treatment response, so may yet prove scalable in coming years. However, this approach would require major investment in technology, training, and expertise to implement on a large scale. Furthermore, development of harmonized processes across studies regardless of sponsor for biopsy and clinical data (for example endoscopy and histology) and integrated analysis with comprehensive clinical parameters (Denson et al. 2019) would permit direct comparison of results and across groups of patients which is currently nor possible due to subtle differences in patients groups and endpoint definitions.

Alternatively, a less technically demanding and cheaper quantitative polymerase chain reaction (qPCR) of a small number of the most importantly identified transcripts would potentially be scalable and could be rapidly turned around in a clinically relevant time limit. qPCR is already embedded in routine clinical practice (e.g., hepatitis C quantification), so it is likely that a qPCR-based technique could be adopted in clinical practice in many units with existing infrastructure. qPCR based platforms can also be used to profile SNPs at low cost and with relative ease.

A major challenge in the field is the development of biomarker platforms that are ready for transposition into clinical practice. Most of the studies described have only been performed in single centres, often using small cohorts. Whilst promising as initial observations, for these platforms to be adopted into clinical practice larger independent validation is required. Furthermore, evaluation in a biomarker randomized control trial and assessment of cost effectiveness and clinical utility are all necessary steps required before it can be used in clinical practice.

There is also a responsibility of research funding bodies to recognise the importance of biomarker validation (as well as discovery), and to look to support robustly designed studies in this area. Now that IBD is becoming an increasingly crowded therapeutic

space, there may be pressures from regulatory agencies, including the FDA and EMA, to encourage the use of companion diagnostics to regulatory approval.

Furthermore, whilst individual biomarkers show promise, the possibility of using composite methods where different clinical, endoscopic (including confocal laser endomicroscopy), genetic, transcriptional, and immunological parameters are combined, might construct a truly personalised medicine approach to treating IBD. A study from Boston described how exploitation of genotyping in combination with robust clinical phenotyping, could be used to predict non-response to infliximab, compared with a clinical only model (Barber et al. 2016). A further example comes from a multi-centre US and Canadian inception cohort study who enrolled 913 newly diagnosed paediatric patients with CD and found that a mulit-omic model incorporating clinical phenotype, ileal gene expression and 16S analysis demonstrated an ability to predict CD complications (Kugathasan et al. 2017). More advanced models such as integrative personal omics profiling (iPOP) (Li-Pook-Than and Snyder 2013), can be developed to include multiple environment, genetic, transcriptional, proteomic, metabonomic and microbiome data which has the potential to make even better predictive models. Therefore, it seems feasible that in the next 5 years a newly diagnosed IBD patient may have a far greater panel of investigations including colonic and blood transcriptional profiling and microbiota composition analysis routinely performed to build a comprehensive model to determine an individual patient's risk of

surgery, likelihood of responding to biologic and small molecule therapies as described in figure 1.10. This would require different working models in IBD clinics with additional expert input from bioinformatics and clinical genetics to join the multi-disciplinary team. The continuing expansion of sophisticated therapeutics available to treat IBD has not only delivered a greater repertoire of options, but also created new opportunities for us to learn smarter ways of deploying these different agents for the benefit of our patients.

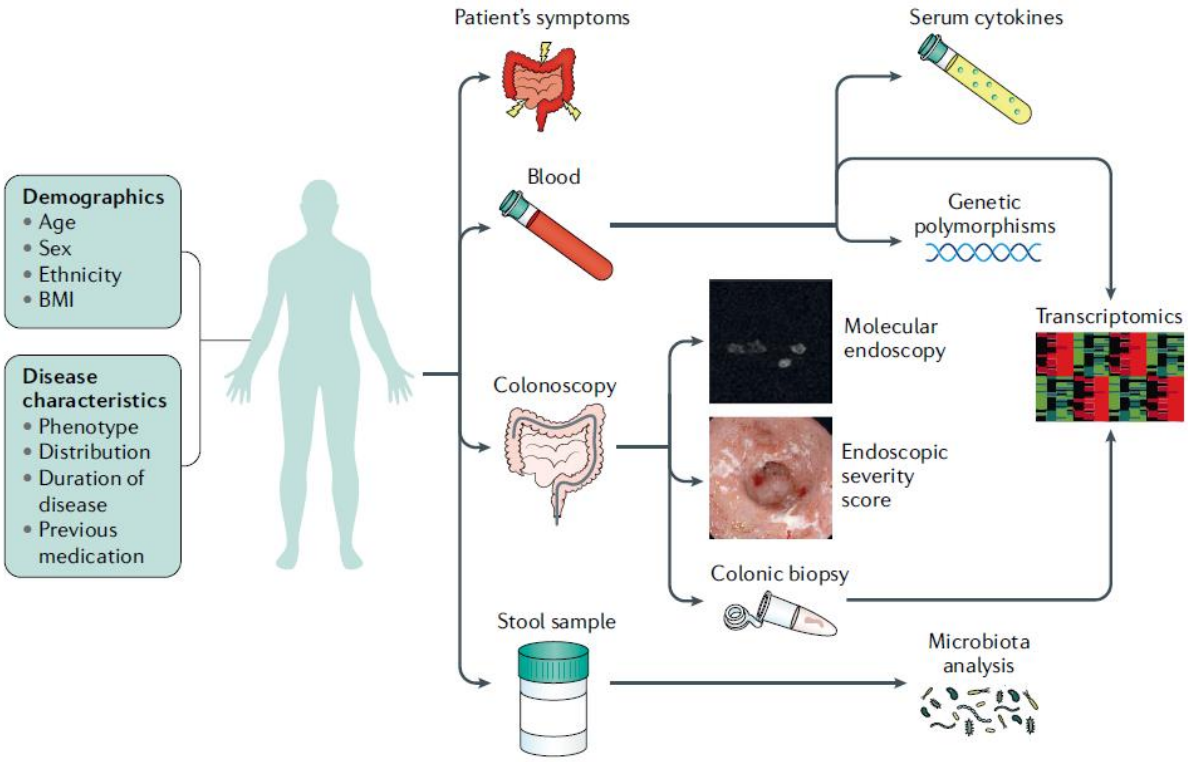


Figure 1.10. Proposed investigations for future personalized medicine in IBD. Potential panels of investigations are shown that might be used in parallel to form a composite score to guide clinicians towards a specific treatment suited to the individual patient. A summary of disease characteristics, demographics, endoscopic severity, and symptoms (current practice) might be combined with innovative technologies of transcriptomic analysis of blood and colonic tissue, gut microbiota analysis, molecular endoscopy, and genetic polymorphisms. (Digby-Bell et al. 2020)

Precision medicine is urgently required in IBD to aid decision of treatment of IBD patients such that the first treatment is the best treatment to reduce time to remission, reduce need for steroids, reduce side effects from ineffective drugs and to better use our scarce financial resources.

The field of transcriptomics is expanding rapidly with the cost of RNAseq dropping to an affordable level (approx. $200 a sample) and expertise in bioinformatics is becoming more accessible and clinically minded. Therefore, we chose to use transcriptomics as our platform as a novel, understudied area in IBD, which has the potential to revolutionise practice.

2 Hypothesis

Our hypotheses are:

1. Exposure of colonic biopsies and laminar propria mononuclear cells from active UC to IL-23 will produce pathologically relevant transcriptional networks

2. IL-23 derived transcriptional profiles will be upregulated in active UC and will be upregulated in patients who have not responded to anti-TNF therapies and those who respond to IL-23 pathway inhibiting drugs

3. Method

To evaluate the hypothesis that IL-23 will induce transcriptional changes in UC we used two models: laminar propria mononuclear cells (LPMCs) culture and whole colonic biopsy (explant) culture. Output from these 2 models was via real time quantitative polymerase chain reaction (RT-qPCR) and RNA sequencing (RNAseq). See Figure 3 for an illustrative explanation.

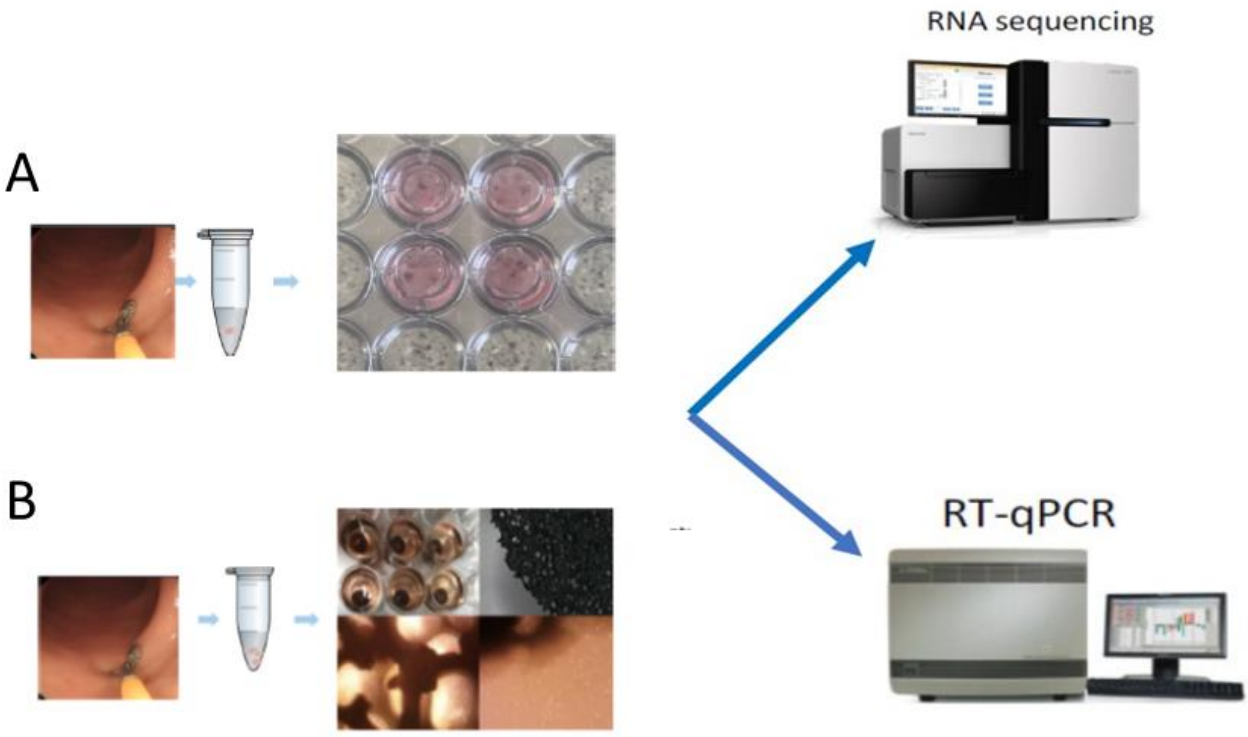


Figure 3 the experimental design for the project.

A – Explants: colonic biopsies placed into a plate containing culture media with the presence or absence of disease relevant cytokines followed by RNA extraction.

B – Lamina propria mononuclear cells (LPMCs): colonic biopsies placed onto cell foam grids to extract lamina propria mononuclear cells which are subsequently cultured in the presence or absence of cytokines.

Output for all the experiments A & B was RT-qPCR and RNAseq

The explant technique was inspired by previous work by Monteleone and colleagues (Monteleone et al. 1999) who demonstrated histological and cytokines changes induced by exposing foetal gut samples to anti-CD3 antibody and IL-12. Similarly work by Rovedatti and colleagues (Rovedatti et al. 2009) demonstrated that exposing LPMCs to anti-CD3 antibody induced IFNγ and IL-17 as measured by ELISA. We postulated that using a similar technique, by exposing colonic mucosal biopsy and LPMCs to IL-23, we will induce transcriptional changes.

3.1. Acquisition of colonic biopsies

Colonic biopsies were obtained from patients with active UC from both Guy's & St Thomas' NHS Hospitals and King's College Hospitals in London. Blood and colonic mucosal samples were taken as part of routine clinical care i.e., endoscopy was not performed for the purpose of research. The study protocol was approved by the R&D departments at each clinical site involved, by the National Institute for Health Research- Research Ethics Committee (REC reference: 15/LO/1998) and was added to the South London Clinical Research Network (CRN) portfolio studies.

The diagnosis of UC was based on the clinician's assessment and on the opinion of the attending gastroenterologist and the assessment of clinical information and previous

endoscopic and histological findings. Endoscopic severity was based on the endoscopist's assessment.

Patients who attended for routine colonoscopy for the indications: rectal bleeding or iron deficiency anaemia and in whom no inflammation was identified endoscopically were included as healthy (non-inflammatory) controls. Relevant patient clinical data and laboratory parameters were collected at the time of recruitment and extracted from the electronic patient records using the pre-designed patient information form which Phil Tomlinson and I created (figure 3.1). Data was anonymised using a code specific using the initials of the investigator and the sequential number of the patient recruited, e.g., JDB48. This data was transferred to a rolling excel spreadsheet held under password on a Guy's & St Thomas.' Once the data was transferred to the excel the paper sheet was destroyed.

PREDICTING RESPONSE TO BIOLOGICS IN IBD - Clinical Data Form (v1.7)

Date completed _____

Patient ID _____ **D.O.B.** _____

Surname _____ **Gender** _____

First name _____ **Hospital number** _____

Ethnicity

☐ Caucasian ☐ South Asian ☐ East Asian ☐ Hispanic ☐ African/Caribbean ☐ Jewish

☐ Other please specify _____

For all, country of origin _____

☐ **Family history of IBD**

Relative	Phenotype	Hospital

Smoking status: ☐ Never ☐ Current ☐ Ex-smoker Number of pack years _____

Month and year of diagnosis _____/_____

☐ **Crohn's Disease** Montreal classification

☐ Ileal ☐ Colonic ☐ Ileo-colonic

☐ Inflammatory ☐ Stenotic ☐ Perforating

☐ Perianal ☐ Upper GI ☐ OFG

☐ **Ulcerative Colitis** Extent of disease

☐ Proctitis ☐ Left-sided ☐ Extensive

Start date of this biologic treatment _____

Previous biologic 1 ☐ Infliximab ☐ Adalimumab ☐ Vedolizumab

Start date _____ Length of treatment _____ months

Reason stopped ☐ Primary non response ☐ Loss of response ☐ Infusion reaction ☐ Intolerance ☐ Antibody detection ☐ Remission

Previous biologic 2 ☐ Infliximab ☐ Adalimumab ☐ Vedolizumab

Start date _____ Length of treatment _____ months

Reason stopped ☐ Primary non response ☐ Loss of response ☐ Infusion reaction ☐ Intolerance ☐ Antibody detection ☐ Remission

☐ PMH of malignancy Significant PMH _____ ☐ Does patient want to receive publication?

PREDICTING RESPONSE TO BIOLOGICS IN IBD - Clinical Data Form (v1.7)

HBI _____ SCCAI _____

Latest endoscopic Mayo score (0-3) _____ ☐ Histological evidence of CMV infection

Weight _____ kg Height _____ cm BMI _____

CRP _____ Date _____ Faecal calprotectin _____ Date _____

☐ Current oral mesalazine Drug name _____ Dose _____

☐ Current thiopurines Drug name _____ Dose _____

Last TGN result _____ Date _____ Lifetime length _____ months of thiopurines

☐ Current methotrexate Drug name _____ Dose _____

☐ Current suppositories / enemas (mesalazine or steroid) Drug name _____ Dose _____

☐ Oral steroids Drug name _____ Dose _____

If YES: ☐ Stable dose ☐ Reducing course

Current biologic ☐ Infliximab ☐ Adalimumab ☐ Vedolizumab Time since last dose _____ days

Dose _____ mg/kg Dose interval _____ weeks Drug level _____ Date _____

Details of sample(s) taken ☐ Blood Sample ID _____

☐ Colonic biopsy Sample ID _____

Other comments

Signature _____

Figure 3.1. demographics and disease information form

Biopsies were taken from the left colon between 20-30cm in every patient to reduce variance. Of note there is evidence that there are gene expression changes in samples taken from right versus left colon (Noble et al. 2008).

We used a Mayo score of 0 and 1 being inactive disease and 2 and 3 being active disease which is the commonly accepted cut off in the majority of recent clinical trials (Sandborn et al. 2017; Feagan et al. 2017; Vermeire et al. 2014) Example endoscopic images of Mayo scores can be seen in Figure 1.3.1.

The Mayo scoring system is not without its issues however especially in differentiating Mayo 1 and 2 disease with significant variation in classification even amongst 'experts' with κ=0.75 (Walsh et al. 2014). A newer, ratified scoring and is being adopted across multiple centres in the UK and worldwide called the UC Endoscopic Index of Severity (UCEIS) (Travis et al. 2012). This system has the advantage of being less subjective therefore allowing better inter-observer agreement which is helpful in the trial setting as well as in clinical practice. The correlation coefficient between the UCEIS and overall severity evaluation was 0.94 ($p<0.0001$), meaning it accounts for 88 % of the variance in overall assessment of severity between observers (Travis et al. 2013). The UCEIS was adopted at Guy's & St Thomas' at the start of 2017 (i.e., midway through the project) and so it was not

used for the purposes of this study but if the study were to be validated then using this score would be suggested.

I developed two standard operating procedures (SOPs). My aim was to aid sample collection when I was not present for colonic explant and LPMC generation to ensure all necessary steps were taken and to reduce variance.

3.2. Lamina propria mononuclear cells

The traditional technique for extracting LPMCs was to 'digest' them using a combination cutting the biopsies into very small pieces using a scalpel then placing the biopsies into a suspension of DNase and collagenase to extract LPMCs (Okazawa et al. 2002; Di Sabatino et al. 2007). As a lab this was the standard technique when I joined in 2015 but was notorious in that it was time consuming, operator dependent and gave a variable number of LPMCs. My colleague Robin Dart in Adrian Hayday's lab was performing similar experiments using LPMCs, but he was using a new techniques using cell foam matrices (Di Marco Barros et al. 2016). They had adapted a technique for obtaining lymphocytes from dermal tissue and had found higher yield of LPMCs (data not included). Whilst the techniques were compared by numerical numbers of cells extracted they were not analysed as to the composition of cells. I recognise this as a limitation of the technique but we used the technique throughout the experiments and

so we were consistent with this approach. Thus, I adopted this technique which was used throughout the study.

The full protocol is below but in brief the colonic biopsy is crushed gently by inverting the cell foam matrix and then placed in a well of 15 well plate and covering them with culture media and IL-2. After 48 hours the cells are harvested and then placed in a 96 well plate with culture media in the presence or absence of IL-23.

The SOP to produce LPMCs is seen below.

LPMC isolation from colonic biopsies using grids

Equipment required prior to endoscopy:

- 1.5ml Eppendorf containing 1ml HBSS stored at 4C
- Yellow top serum tube
- Blue topped Tempus tube
- 'Defining immune pathways driving gut inflammation' version 2 date 29.01.2016 consent form and patient information sheet

Consent patient using 'Defining immune pathways driving gut inflammation' version 2 date 29.01.2016 to take colonic biopsies and a blood sample

Up to 18 biopsies (1 per pass of biopsy forceps) taken from rectosigmoid (15-20cm from anus). Place 1st biopsy into cryovial containing 600µl of RNAlater (ThermoFisher, UK) and ensure it is submerged then place at 4°C until transfer. Using a new biopsy forceps place the remaining biopsies into 1.5ml Eppendorf containing 1ml HBSS kept at 4C until use.

Label Eppendorf with pre-assigned research code e.g., JDB32 and place in pathology samples bag and keep at 4°C or on ice until collection / transfer

Blood tests taken post procedure (either with fresh venepuncture or from withdrawal of blood from the cannula after first 10ml has been discarded)

1st – Yellow topped serum tube

2nd - Blue topped Tempus tube – **requires inverting for 15 seconds after blood added**

Samples labelled with pre-assigned research code e.g., GST 32

Samples placed in pathology bag and kept at room temperature until collection / transfer.

If at Guy's, the endoscopist should contact researcher directly to collect samples

If sample acquired at St Thomas,' then sample should be transferred to Guy's by HTA approved method.

Once samples received in laboratory:

Eppendorf containing colonic biopsies to be removed from pathology specimen bag and placed on ice until processed

Tempus tube to be kept at RT for 24 hours then transferred to -20°C.

Serum tube centrifuged at 2000rpm for 5 minutes, then serum removed with Pasteur pipette equally into 2 pre-labelled 1.5ml Eppendorf and stored at -20C.

Eppendorf containing biopsies to have HBSS discarded, and biopsies transferred onto petri dish containing 10ml 'wash' media (see below for recipe) for 10 minutes at RT.

Count the number of biopsies and place the same number of grids in a 15ml falcon flask containing 10ml PBS + 2.5ul (100mcg/ml) rat tail collagen (400mg/ml from BD Biosciences) for 10 minutes in a water bath at 37°C

Make up Gut-T media (see below). Put 2.5ml x number of grids (i.e., if 10 grids put 25ml into a 50ml Falcon tube) + IL-2 solution (100U/ml)

Pipette 2ml of Gut-T + IL-2 solution into each well intended to be used

Discard collagen solution and wash grids twice with PBS.

Place grids on upturned lid of petri dish

Individually remove a biopsy using forceps and place onto a grid. Turn the grid over and squash it into the petri dish then turn it over so the biopsy is facing up

Then place the grid into a wells of a flat bottomed 24 well plate which have already been filled with media + IL-2

Place in incubator at 37°C for 48 hours

Harvesting

Add 10ml of HBSS and 0.01M HEPES in 15ml falcon tube

Remove plate from incubator and place grids in 15ml falcon with HBSS + HEPES

Place 70um filter on top of 50ml falcon flask

With Pasteur pipette aspirate supernatant and pass it through filter into 50ml flask

Wash wells x2 with HBSS + HEPES and pass-through filter

Agitate 15ml falcon containing grids then pour through filter into 50ml falcon through filter

Individually pick up grids and aspirate fluid from them with a Pasteur pipette then pass fluid through filter

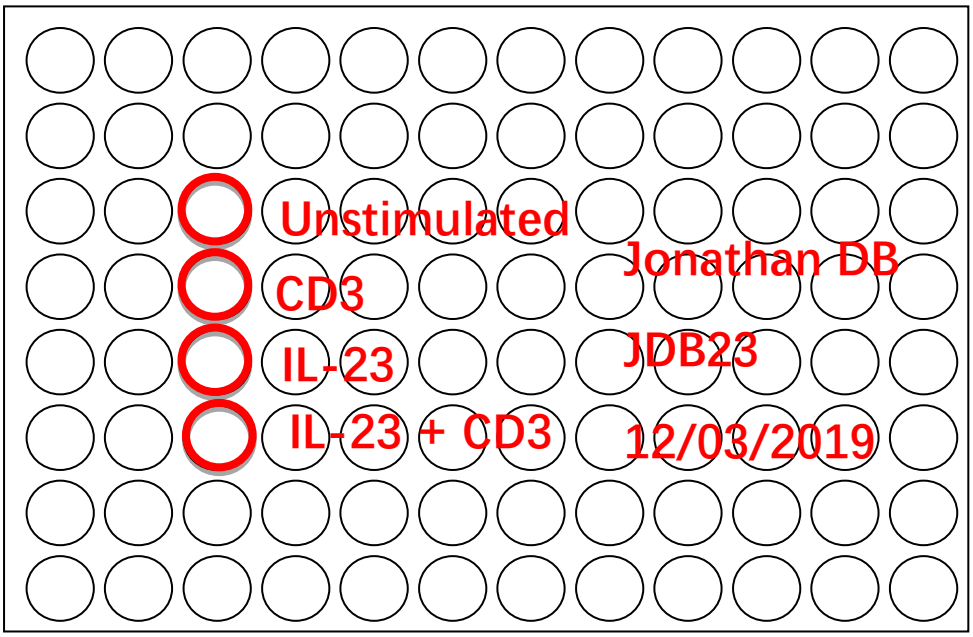
Centrifuge at 1600rpm for 5 minutes

Discard supernatant and resuspend cells in 1ml.

Perform cell count using haemochromocytomter

Resuspend cells in 2,000,000 per ml of Gut-T media

Plate design will depend upon the number of cells



4-hour RNA plate

200,000 cells per well (100ul) then add cytokine **'DOUBLE STRENGTH'.** To explain: the cell suspension has a volume of 100ul without cytokine so to achieve a given concentration double the intended final concentration is needed to be added in half the volume of the final volume.

If an excess of cells, then use multiple wells for each condition.

Place in incubator at 37C with 5% CO2 for 4 hours

At 4 hours remove plate from incubator

Aspirate supernatant up and down to ensure mixing then place into 1.5ml RNase free Eppendorf. Wash well x2 with wash media. Examine well under the microscope to see if cells remain in well. If so, then add more wash media.

Centrifuge for 1600rpm for 5 minutes.

Remove supernatant into pre-labelled Eppendorf and place on ice until transfer to -20° C.

Add 800ul Qiazol (Qiagen, Germany) to the RNase free Eppendorf with a pellet if cells. Then homogenate with 20G needle and 1ml syringe x10

Place samples at -80C

Wash media

RPMI 1640

10% FCS

Penicillin 500U/ml

Streptomycin 500ug/ml

Gentamicin 100ug/ml

Amphotericin B 12.5ug/ml

Metronidazole 5ug/ml

Gut-T media

RPMI 1640

10% FCS

L-glutamine 292ug/ml

Penicillin 100U/ml

Streptomycin 100ug/ml

2-mercaptoethanol 3.5ul/L

Gentamicin 20ug/ml

Amphotericin B 2.5ug/ml

Metronidazole 1ug/ml

3.3. Colonic explants

In brief the whole biopsies are placed into a 96 well plate with 1 biopsy placed into a single well, with culture medium in the presence or absence of IL-23 then they are then placed at 37°C for 4 hours. The samples were removed from the incubator and the supernatant was collected and stored at -80°C. The biopsy was placed into RNAlater and homogenised using an electric pestle and mortar and with a needle and syringe.

Standard Operating Procedure for obtaining colonic biopsies for explant experiments

Equipment required prior to endoscopy:

- 1.5ml Eppendorf containing 1ml HBSS stored at 4C
- Yellow top serum tube
- Blue topped Tempus tube
- 'Defining immune pathways driving gut inflammation' version 2 date 29.01.2016 consent form and patient information sheet

Consent patient using 'Defining immune pathways driving gut inflammation' version 2 date 29.01.2016 to take colonic biopsies and a blood sample

8 biopsies (1 per pass of biopsy forceps) taken from rectosigmoid (15-20cm from anus). Place 1st biopsy into cryovial containing 600ul of RNA later and ensure it is submerged. This cryovial should be kept at 4C for 24 hours then placed at -20C. Place remaining biopsies into 1.5ml Eppendorf containing 1ml HBSS kept at 4C until use.

Label Eppendorf with pre-assigned research code e.g., JDB32 and placed in pathology samples bag and kept at 4C or on ice until collection / transfer

Blood tests taken post procedure (either with fresh venepuncture or from withdrawal of blood from the cannula after first 10ml has been discarded)

1st – Yellow topped serum tube

2nd - Blue topped Tempus tube – **requires inverting for 15 seconds after blood added**

Label samples with pre-assigned research code e.g., GST 32

Samples placed in pathology bag and kept at room temperature until collection / transfer.

If at Guy's, the endoscopist should contact researcher directly to collect samples

If sample acquired at St Thomas,' then sample should be transferred to Guy's by HTA approved method e.g., courier

Once samples received in laboratory:

Eppendorf containing colonic biopsies to be removed from pathology specimen bag and placed on ice until processed

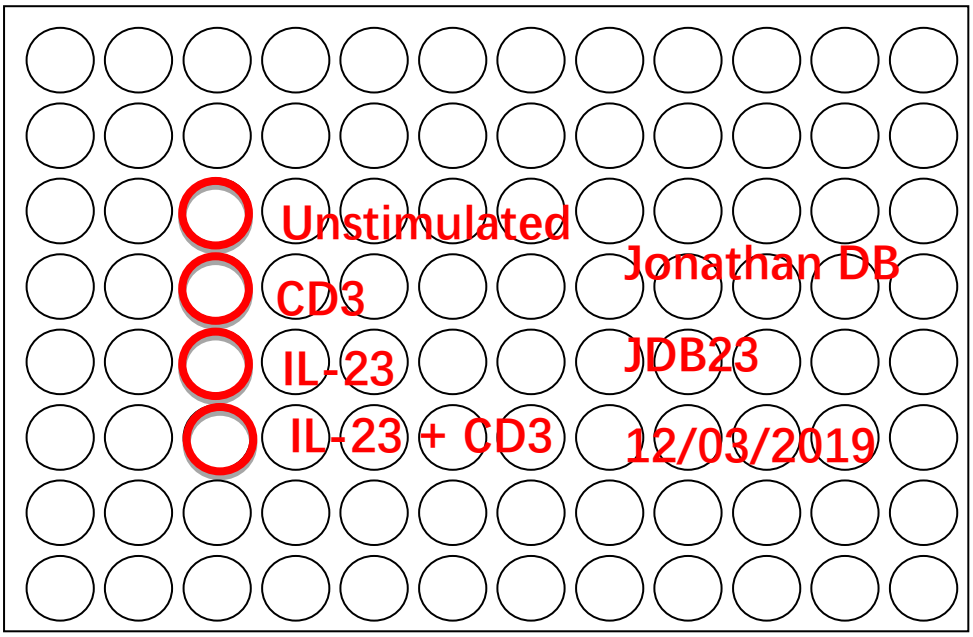
Tempus tube to be kept at room temperature for 24 hours then transferred to -20C.

RNA later biopsy to be stored at 4°C for 24 hours then transferred to -20°C.

Serum tube centrifuged at 2000rpm for 5 minutes, then serum removed with Pasteur pipette equally into 2 pre-labelled 1.5ml Eppendorf and stored at -20C.

Eppendorf containing biopsies to have HBSS discarded, and biopsies transferred onto petri dish containing 10ml 'wash' media (see below for recipe) for 10 minutes at RT

Label 96 well round bottomed plate and label 4 wells away from the edge as well as name of researcher, patient ID, and date



Individually place a biopsy in the assigned well on a 96 well plate and cover with 150ul of Gut-T media (see below for recipe) containing relevant cytokine. Aliquots of 200ul pre-made media and cytokine can be found in 4C fridge

8th biopsy is a spare in case of error in previous step

Place in incubator at 37C with 5% CO2 for 4 hours

At 4 hours remove plate from incubator

Remove supernatant into pre-labelled Eppendorf and place on ice until transfer to -20°C

Place biopsy into pre-labelled 1.5ml RNase free Eppendorf and add 800ul Qiazol (Qiagen, Germany). Macerate/disrupt biopsy with electronic pestle and mortar for 30 seconds or until no visible sample. Then homogenate with 20G needle and 1ml syringe x10

Place samples at -80C

Wash media

RPMI 1640

10% FCS

Penicillin 500U/ml

Streptomycin 500ug/ml

Gentamicin 100ug/ml

Amphotericin B 12.5ug/ml

Metronidazole 5ug/ml

Gut-T media

RPMI 1640

10% FCS

L-glutamine 292ug/ml

Penicillin 100U/ml

Streptomycin 100ug/ml

2-mercaptoethanol 3.5ul/L

Gentamicin 20ug/ml

Amphotericin B 2.5ug/ml

Metronidazole 1ug/ml

Cytokine recipes

Cytokine will be made in batches in advance and stored at 4C until used

Unstimulated Gut-T media only

IL-23 Gut-T media + 10ng/ml IL-23

Cytokine	Company & cat no.	Starting concentration	Final concentration
IL-23	R&D 1290-IL	10ug/ml	10ng/ml

3.4. Cytokine choice and dose

We chose to stimulate the explants and LPMCs with IL-23. As aforementioned in the introduction IL-23 is a key cytokine in the pathogenesis of inflammation in IBD and has been identified as a key causative pathway, which is responsible for a proportion of anti-TNF non-response (Schmitt et al. 2018; Duerr et al. 2006). Furthermore ustekinumab (anti-p40 monoclonal antibody targeting both IL-12 and IL-23) is effective in UC and has now in routine clinical use (Sandborn et al. 2019) as well multiple anti-IL23p19 drugs are in phase III clinical trials (risankizumab, brazikumab, guselkumab, mirikizumab). Therefore, examining the effect of IL-23 at a transcriptomic level in colonic immune cells and biopsies may provide key insights into UC pathogenesis and may create biomarkers to aid prediction of response to therapies.

We debated the conditions and length of stimulation for the LPMCs and explants. Prior experiments had stimulated explants and LPMCs for 48 hours (Monteleone et al. 1999; Vossenkamper et al. 2014; Rovedatti et al. 2009) but the output was detection of proteins using ELISA rather than transcriptomic output.

We postulated that the likelihood was that to capture RNA peaks we would require a much shorter stimulation. We considered many factors for and against a short (i.e., 1 hour) to long (i.e., > 24 hour) stimulation. We considered:

- cytokine supply exhausted by the cells during a long experiment
- longer experiments will undoubtedly lead to more cell death which would certainly alter the results
- stimulating too short could be too early to detect meaningful transcriptomic change

At a meeting in early 2017 with Nick Powell, Nat Prescott and Esperanza Perucha and myself we agreed on a stimulation of 4 hours which we considered a sensible balance between stimulating for long enough to induce transcriptional changes within the cells but not too long such that cell death will become predominant and therefore cloud the results.

Dose of cytokine was based upon prior work performed in the laboratory.

3.5. RNA extraction

RNA extraction from Qiazol was performed using the Qiagen RNeasy mini kit with DNase 1 treatment.

We debated the merits of DNase treatment amongst learned colleagues with an interest and experience of RNAseq. We optimised the protocol and decided on using x5 the standard concentration of DNase as well as using an on column clean up. We found that the higher concentration of DNase that was used led to a decrease in contaminant DNA but also led to a reduction in residual RNA. X5 standard concentration struck the balance between greater removal of contaminant DNA but whilst preserving sufficient RNA such that decent quality RNAseq could be performed. Results of experiment comparing DNase concentrations on DNA and RNA levels on the sample as well as RIN scores are shown in figure 3.5.

Samples		Qubit DNA	Total DNA in 20ul	% DNA removed	Qubit RNA	Total RNA in 20ul	% RNA recovered	RIN
D RNA later immediate	On ice	63.60	* 254.4	-	858.0	* 3432	-	6.80
	1x DNase	11.10	222	12.74	133.0	2660	77.51	7.80
	5x DNase	13.20	264	-3.77	134.0	2680	78.09	7.70
	10x DNase	8.68	173.6	31.76	107.0	2140	62.35	7.70
F RNA Later 2-hour delay	On ice	46.00	* 184	-	430.0	* 1720	-	7.50
	1x DNase	2.76	55.2	70.00	57.4	1148	66.74	7.90
	5x DNase	1.35	27	85.33	36.4	728	42.33	8.20
	10x DNase	1.21	24.2	86.85	33.8	676	39.30	8.20
J 4-hour culture in media	On ice	45.20	* 180.8	-	482.0	* 1928	-	6.10
	1x DNase	3.84	76.8	57.52	62.8	1256	65.15	6.60
	5x DNase	2.22	44.4	75.44	49.8	996	51.66	6.80
	10x DNase	1.47	29.4	83.74	36.8	736	38.17	6.70

Figure 3.5 table of comparisons of DNase concentrations effect upon DNA, RNA and RIN

Protocol: On column DNase I Treatment & RNA Clean up

Materials

Materials	Company
RNeasy Mini Kit	Qiagen
RNase-Free DNase Set	Qiagen
Ethanol (molecular grade)	Sigma or equivalent

NB: The following procedure has been adapted from the RNeasy Mini Handbook 06/2012 pg 54-55 and pg 67-68.

Important points

- A maximum of 100ug RNA can be used per column.
- All centrifugations performed at RT (20-30˚C).
- Add solutions directly onto column membrane.

Preparation

- Book Qubit and Bioanalyzer for quantification of DNA, RNA and RIN scoring if required. *iLABs, BRC Genomics Research Platform, 7th floor Tower Wing. Trained users only.*
- Thaw RNA samples on ice. Can take up to an hour.
- Before using buffer RPE for the first time, add 4 volumes of ethanol (96-100%) as indicated on the bottle.
- Prepare DNase I stock solution if needed:
    1. Dissolve DNase I (1500 Kunitz units) in 550µl of RNase-free water using a syringe and needle. Do not open the vial. Mix gently by inverting. Do not vortex.

2. Aliquots (for 1 sample at 5X concentration) – Remove lid and aliquot 50µl DNase I stock into each tube. Store at -20˚C for up to 9 months. Thawed aliquots can be stored at 2-8˚C for up to 6 weeks. Do not refreeze after thawing.

- Label 1.5ml collection/storage tubes (supplied with RNeasy kit) for sample storage.

Procedure

1. Adjust sample to 100µl with RNase-free water. Add 350µl Buffer RLT and mix by pipetting.
2. Add 250µl ethanol (96-100%). Mix by pipetting.
3. Transfer sample (700µl) to a RNeasy Mini spin column placed in a 2ml collection tube (supplied). Close lid, and centrifuge for 15 s at ≥8000 x g (≥10,000rpm). Discard flow-through and tap collection tube on tissue to remove excess flow-through.
4. Add 350µl Buffer RW1 to the spin column. Close lid, and centrifuge for 15 s at ≥8000 x g (≥10,000rpm). Discard flow-through and tap collection tube on tissue to remove excess flow-through.
5. Add 30µl Buffer RDD to 50µl DNase I stock solution. Mix by gently inverting the tube. Do not vortex. Briefly centrifuge. *Buffer RDD supplied with the RNase-Free DNase Set.*
6. Add the DNase I incubation mix (80µl) directly to spin column membrane. Incubate at RT (20–30°C) for 15min.
7. Add 350µl Buffer RW1 to the RNeasy spin column. Close lid, and centrifuge for 15 s at ≥8000 x g (≥10,000rpm). Discard flow-through.

NB: Ensure that ethanol is added to Buffer RPE before use.

8. Add 500µl Buffer RPE to the RNeasy spin column. Close lid, and centrifuge for 15 s at ≥8000 x g (≥10,000rpm) to wash. Discard the flow-through.
9. Add 500µl Buffer RPE to the RNeasy spin column. Close lid, and centrifuge for 2min at ≥8000 x g (≥10,000rpm) to wash.

NB: Residual ethanol may interfere with downstream reactions. If needed, wipe any residual flow-through from outside of spin column.

10. Place the RNeasy spin column into a new 2ml collection tube and discard the old collection tube. Close lid, and centrifuge at full speed for 1min.
11. Place the RNeasy spin column into a new 1.5ml collection/storage tube (supplied). Add 20–30µl RNase-free water directly to spin column membrane. Close lid, and centrifuge for 1min at ≥8000 x g (≥10,000rpm) to elute the RNA.
12. Transfer eluate back onto column membrane and centrifuge for 1min at ≥8000 x g (≥10,000rpm).
13. Keep samples on ice and continue to DNA/RNA quantification, and RIN scoring.

For long-term storage, store at -80˚C.

DNA/RNA quantification, and RIN scoring

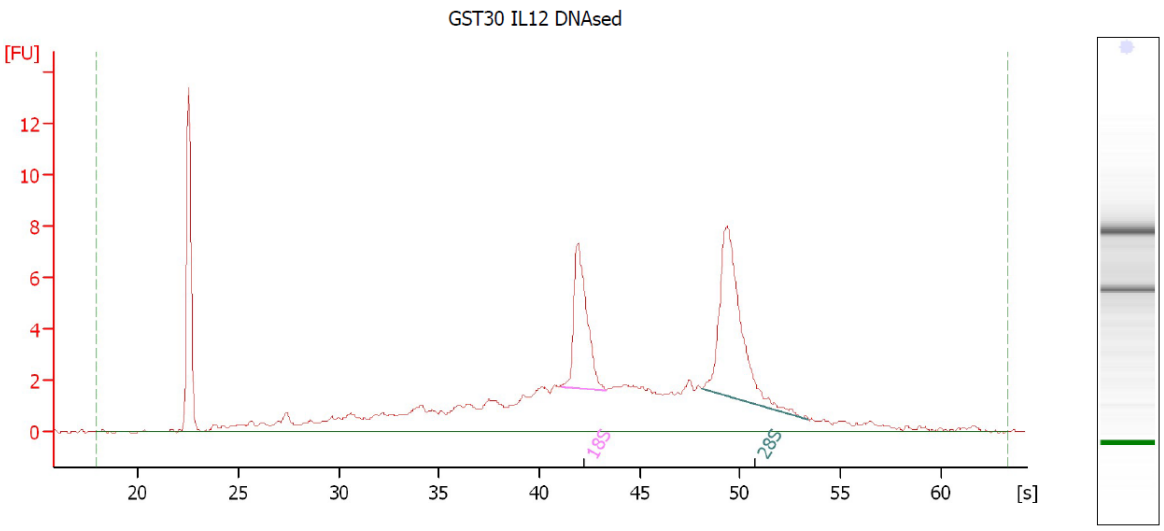
1. Use Qubit for quantification of DNA (Qubit dsDNA HS assay kit) and RNA (Qubit RNA BR assay kit). *See BRC Genomics Facility User Manual – Qubit version (3.0) Fluorometer.*
2. Use Bioanalyzer for RIN scoring. *See Agilent RNA 6000 Nano Kit Quick Start Guide. Assay class: Eukaryote Total RNA Nano.*

3.6. Quality control

Quality of the RNA was performed using Nanodrop, RNA quantification, DNA quantification and RNA integrity number (RIN) score all of which were performed in the Biomedical Research Centre on 7th floor of Guy's Tower. Using Qubit v3 we evaluated both for quantity of RNA using Qubit RNA broad range assay kit and quality of the RNA using Nanodrop aiming for 260:280 ratio of >1.9 and bioanalyser (2100 Bioanalyser System, Aligent USA) with RIN score >6.5.

3.6.1. RIN scores

Determining the integrity of RNA starting materials is a critical step in gene expression analysis. Using electrophoretic separation on microfabricated chips, RNA samples are separated and subsequently detected via laser induced fluorescence detection. The Bioanalyzer software generates an electropherogram and gel-like image. The RIN was developed to remove individual interpretation in RNA quality control. It takes the entire electrophoretic trace into account. The RIN score is based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled, and repeatability of experiments is ensured. Examples from our own work in figure 3.6.1.1 and figure 3.6.1.2 show a good RIN score sample and a poor RIN score sample.



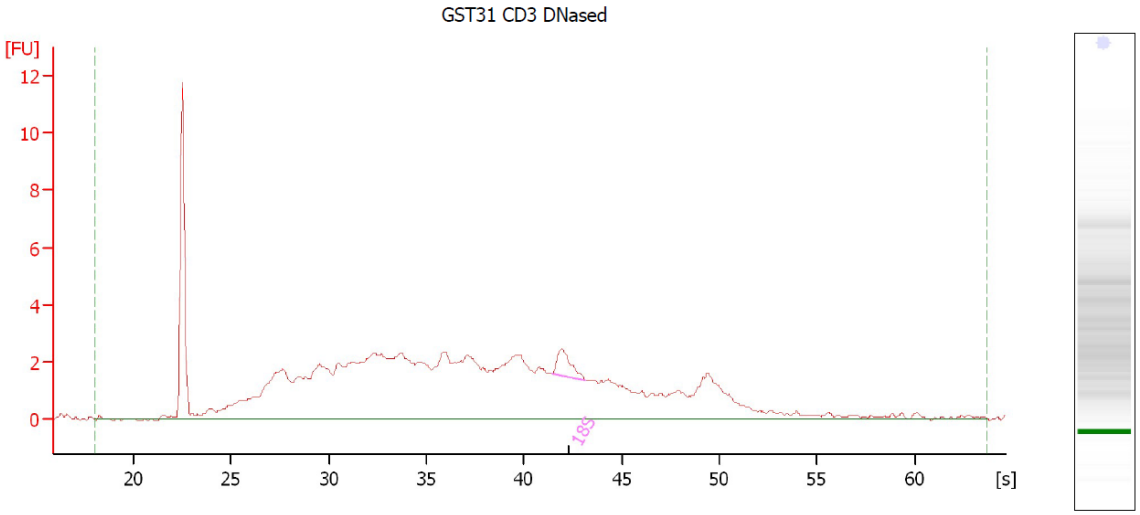
Overall Results for sample 5 : GST30 IL12 DNAsed

RNA Area:	103.1	RNA Integrity Number (RIN):	7.5 (B.02.08)
RNA Concentration:	55 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1.7	Result Flagging Label:	RIN: 7.50

Fragment table for sample 5 : GST30 IL12 DNAsed

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.10	43.30	10.0	9.7
28S	48.12	53.40	16.6	16.1

Figure 3.6.1.1 demonstrates an RNA sample of good quality with a high RIN score with clear peaks of 18S and 28S portions with low expression in the rest of the sample.



Overall Results for sample 8 : GST31 CD3 DNased

RNA Area:	116.5	RNA Integrity Number (RIN):	3 (B.02.08)
RNA Concentration:	62 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	RIN:3

Fragment table for sample 8 : GST31 CD3 DNased

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	41.40	43.15	1.8	1.6

Figure 3.6.1.2 showing bioanalyser report from a sample with poor integrity RNA. Note the barely visible peaks of 18S and 28S with a marked 'left shift' of RNA material which is not present in the superior quality sample.

Once QC had been performed, we aliquoted a portion of the RNA into a separate RNase free tube which could be sent directly for RNA sequencing therefore not requiring the RNA to be thawed which may increase the risk of degradation. The amount aliquoted depended on the quality and quantity of the RNA produced ensuring there was sufficient sample to perform RT-qPCR.

An example table of 20 samples which all had RNA extracted, QC performed and aliquoted is seen in table 3.6.1.3 below. A database of all the samples was kept and managed by Tsui Tjir.

		RNA Analysis			Aliquots (labelling: Sample ID, Treatment, [], Date)	
		NanoDrop	Qubit	Bioanalyzer		
Sample ID	Treatment	260/280	RNA [] (ng/ul)	RIN	RNA (ug)	Vol (ul)
GST35	Unstim	2.04	304.0	7.7	1.20	3.95
GST35	CD3	2.07	111.0	6.3	1.20	10.81
GST35	IL23	2.02	182.0	5.0	1.20	6.59
GST35	IL23+CD3	2.09	102.0	5.7	1.20	11.76
GST37	Unstim	2.05	53.6	6.5	0.65	12.13
GST37	CD3	2.10	117.0	6.8	1.00	8.55
GST37	IL23	2.04	222.0	7.7	1.00	4.50
GST37	IL23+CD3	1.96	57.6	7.0	0.75	13.02
GST52	Unstim	1.86	49.4	7.2	0.84	16.98
GST52	CD3	2.09	74.2	6.7	1.26	16.98
GST52	IL23	1.94	47.0	7.7	0.79	16.81
GST52	IL23+CD3	1.89	116.0	7.0	1.97	16.98
JDB2	Unstim	2.02	220.0	4.9	2.00	9.09
JDB2	CD3	2.04	240.0	7.0	2.00	8.33
JDB2	IL23	2.12	784.0	4.4	2.00	2.55
JDB2	IL23+CD3	2.08	442.0	7.0	2.00	4.52
JDB3	Unstim	2.09	476.0	7.2	2.00	4.20
JDB3	CD3	2.06	196.0	7.2	2.00	10.20
JDB3	IL23	2.08	432.0	6.0	2.00	4.63
JDB3	IL23+CD3	2.09	392.0	6.5	2.00	5.10

Table 3.6.1.3 showing the sample code, treatment of the sample, nanodrop, qubit, RIN score and aliquot details of 20 samples which were run in a single day.

3.7. cDNA synthesis

After an adequate aliquot was taken for RNAseq a proportion the remaining sample was converted to cDNA so that RT-qPCR could be performed.

cDNA was created using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) as per protocol. RevertAid negative and no template control samples were performed in each batch in samples with enough to ensure quality standards of purity were maintained. Samples were placed in a thermocycler for 60 mins at 40°C then 70°C for 5 mins then 18°C until the samples were removed. Normally we created 500ng at a time, but this depended on the total amount of RNA that was created and the desired number of RT-qPCR experiments that we had planned. We performed cDNA synthesis in batches containing all the treated samples from each patient to reduce the variance produced by the batch effect. Once created we diluted the cDNA to 6.25ng/µl and stored it at -20°C for long-term storage.

To minimize freeze and thaw of the RNA with the potential for RNA degradation we created cDNA on the same day that the RNA was extracted and had QC performed.

3.8. RT-qPCR

Using the cDNA as described in the above section we performed RT-qPCR using the Taqman system (ThermoFisher, USA). The following commercially available primers were used:

IL-22	Hs01574154_m1
IL17A	Hs00174383_m1
TNF	Hs00174128_m1
S100A8	Hs00374264_g1
OSMR	Hs00384276_m1
IFNγ	Hs00989291_m1
18S	Hs99999901_s1

After discussion we chose 18S as the housekeeper which has been the much-used standard reference gene in the laboratory for many years and has a reliable record of consistency.

I created a protocol to ensure consistency when performing RT-qPCR. In brief a master mix was created such that in each well there would be:

0.5µl gene of interest

0.5µl reference gene

2µl H20

5µl qPCR master mix (Thermo Fisher, USA)

8µl master mix was placed into each required well of a 384 well plate. Subsequently 2µl of cDNA sample was placed into the well totalling 10µl. They were run on a RT-qPCR cycler at 2mins at 50°C, 10mins at 95 °C, 15sec at 95 °C, 1min at 60 °C for 40 cycles.

Samples were run in triplicate where possible and in duplicate if insufficient sample was available. In triplicate samples were excluded if deemed an outlier.

An example of the qPCR trace can be viewed in Figure 3.8.1.

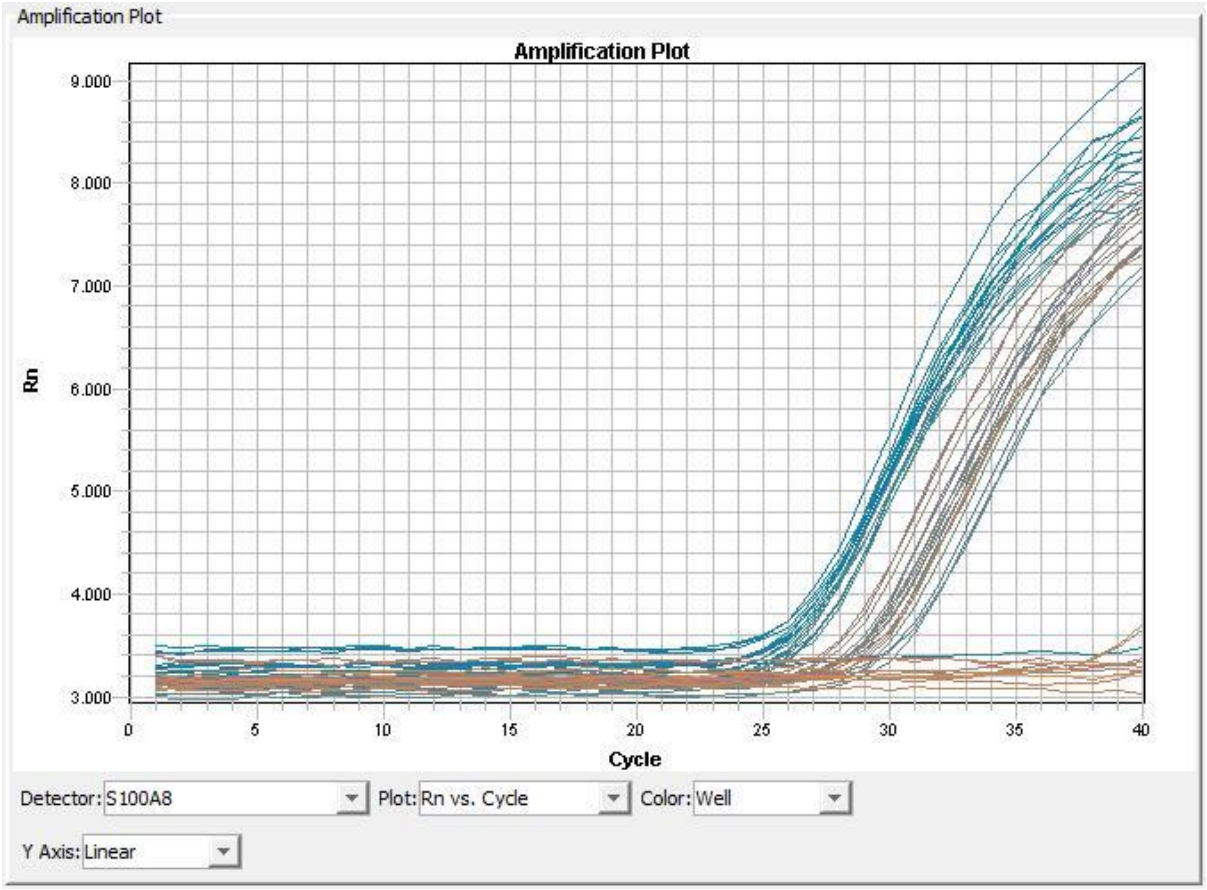


Figure 3.8.1 RT-qPCR amplification plot of gene S100A8 from whole gut biopsy explants. The flat lines are from either no template control (i.e., water instead of RNA) or RevertAid negative samples which provide a negative control. The rest of the samples show a positive result with the lines furthest to the left having the highest expression and the ones to the right lesser expression.

1 2 3

Well	Sample Name	Detector	Ct1	Ct2	Ct3	dCt1	dCt2	dCt3	RA1	RA2	RA3	Mean	SD
1	JDB1 bx unstim	18s	17.0706	17.3248	16.8781	15.2046	14.9724	15.3788	2.6482E-05	3.1108E-05	2.3471E-05	2.7020E-05	3.8468E-06
1	JDB1 bx unstim	IFNg	32.2752	32.2972	32.2569								
4	JDB1 bx unstim	18s	17.4754	17.1070	17.0827	15.1835	14.9570	15.1310	2.6872E-05	3.1441E-05	2.7868E-05	2.8727E-05	2.4029E-06
4	JDB1 bx unstim	IL22	32.6590	32.0640	32.2137								
7	JDB1 bx unstim	18s	17.3229	17.3815	17.2259	13.3588	12.8690	12.9975	9.5192E-05	1.3368E-04	1.2228E-04	1.1705E-04	1.9769E-05
7	JDB1 bx unstim	mTNFa	30.6817	30.2504	30.2234								
10	JDB1 bx unstim	18s	17.1775	17.2038	17.1274	14.8958	14.7829	15.0950	3.2804E-05	3.5473E-05	2.8573E-05	3.2283E-05	3.4793E-06
10	JDB1 bx unstim	OSMR	32.0733	31.9867	32.2224								
13	JDB1 bx unstim	18s	16.9814	17.0884	16.9273	7.1859	7.3038	7.3271	6.8678E-03	6.3292E-03	6.2278E-03	6.4749E-03	3.4397E-04
13	JDB1 bx unstim	S100A8	24.1674	24.3922	24.2543								

1. Calculate dCt by subtracting housekeeper (18s) from the gene of interest (IFNg) from each of the three replicates
2. Calculate Relative expression (RA) by using standard calculation = 2^(-dCT)
3. Calculate mean and standard deviation (SD) of each sample

Figure 3.8.2 worked example of RT-qPCR calculations performed to calculate relative expression of each gene of interest

1. calculate dCt by subtracting the expression of the housekeeper from the expression of the gene of interest.

2. Calculate relative expressions by using the standard calculation =2^(-dCt).

3. Calculate mean and standard deviation of the relative expressions of the three replicates. This allows identification and exclusion of outliers if necessary

We had several discussions regarding the best way to present qPCR data and referred to the MIQE guidelines (Bustin et al. 2009); we decided that expression would be measured as $2^{-(CT_{gene})} / 2^{-(CT_{REF})}$.

3.9. RNAseq

RNA sequencing (RNA-seq) uses high-throughput analysis of the entire transcriptome (all mRNA transcripts present in a particular sample) (Ozsolak and Milos 2010; Wang, Gerstein, and Snyder 2009). Previously to analyse a range of RNA from a sample either large numbers of RT-qPCR is performed which is extremely time consuming or performing microarrays. The limitation of microarrays is that they are limited to a pre-prescribed number of transcripts which inherently excludes potential targets which have not yet been discovered. Furthermore, hybridization techniques have high background noise due to cross hybridization and so subtle changes in RNA quantification may be missed. However, RNAseq has been awfully expensive until the last few years such that it was prohibitive to perform in all but a handful of institutions. Furthermore, analysis is complex and involves the familiarity of complex data modelling and considerable processing power.

RNAseq method in brief (see Figure 3.9.1.): RNAseq involves taking RNA, fragmenting, and then converting it to copy DNA (cDNA) via reverse

transcription with adaptors attached to one or both ends. Each molecule is then sequenced in a high-throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing). Following sequencing, the resulting reads are either aligned to a reference genome to provide quantifiable and qualifiable data.

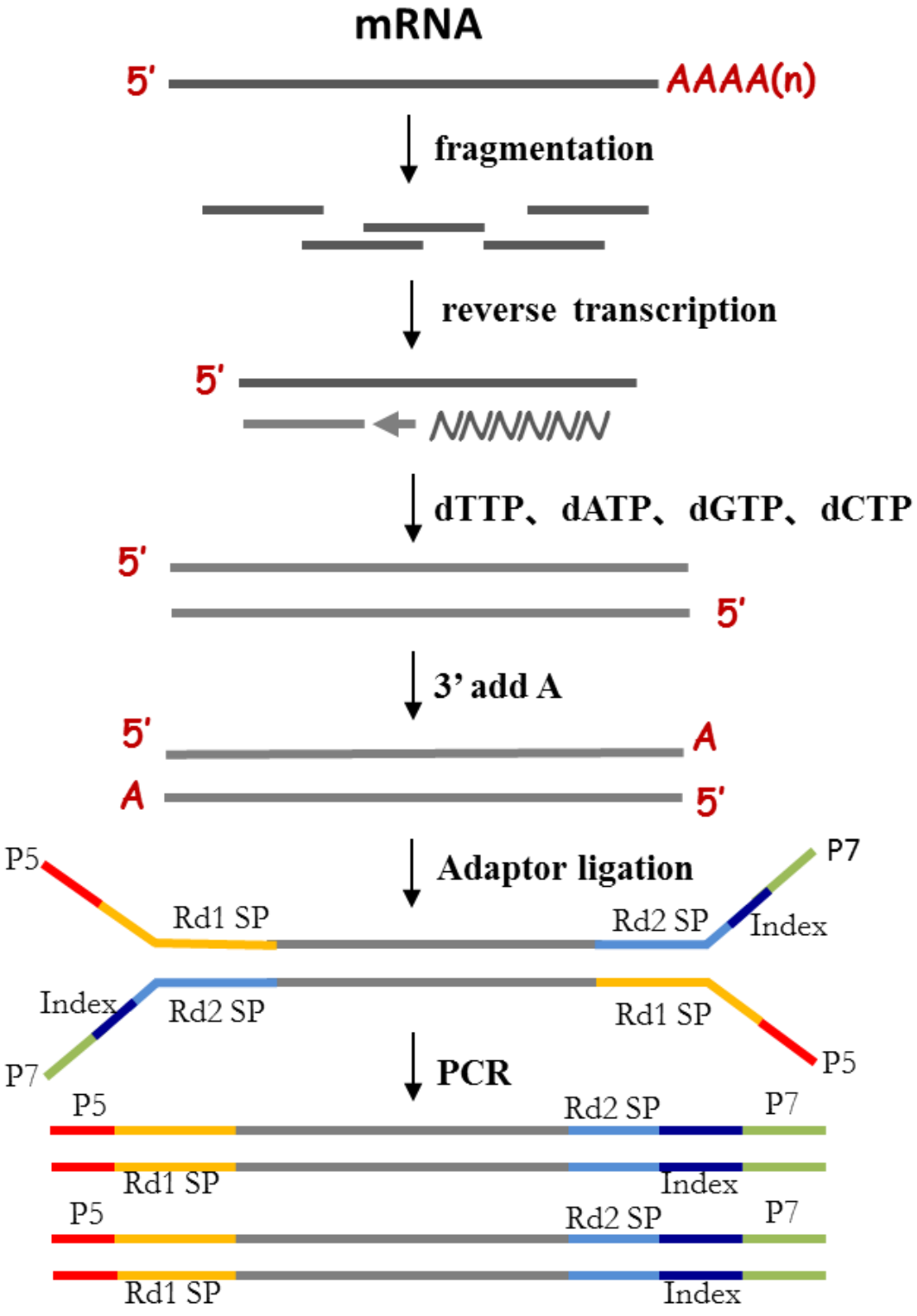


Figure 3.9.1. Image demonstrating steps performed to undertake RNAseq. RNA is fragmented and then amplified (if required). The RNA is then converted into

cDNA using reverse transcription and adaptors added and PCR enrichment performed.

We partnered with Novogene, a Chinese company specialising in high throughput genetic and genomic services. We received quotes for the RNAseq experiments that we wanted to perform from several local and international service providers and Novogene were by far the most competitive in terms of price and could provide timely and decent quality sequencing.

3.10. RNAseq quality control

Adequate quality control (QC) is essential to ensure the experiments are dependable and repeatable. Novogene provided comprehensive quality control data with several aspects as detailed below. One individual QC component is inadequate to ratify quality, but a picture created by QC aspects can identify outliers which can then be excluded from downstream analysis.

Figure 3.10 shows a summary and comparison of the QC performed across all the samples which had RNAseq performed in that run. The main purpose of comparing QC is to identify outlier samples which can then be removed from further analysis. As can be seen from the table there are no clear

outliers, all samples have good read counts (> $x10^7$) and comparable error rates and C/G content.

Sample name	Raw reads	Clean reads	Clean bases	Error rate(%)	Q20(%)	Q30(%)	GC content(%)
unstim_A	58219910	57027764	8.6G	0.02	97.09	92.74	51.96
unstim_B	71291898	67386786	10.1G	0.02	96.48	91.52	52.98
unstim_C	58543126	54806020	8.2G	0.02	96.27	91.39	53.10
unstim_D	58270882	54574940	8.2G	0.02	96.21	91.31	52.49
unstim_E	56201970	52810110	7.9G	0.02	96.45	91.75	53.39
CD3_A	59270186	56491946	8.5G	0.02	96.77	92.16	51.97
CD3_B	61287072	57813486	8.7G	0.02	96.61	91.83	52.81
CD3_C	47288428	44364124	6.7G	0.02	96.26	91.42	52.07
CD3_D	44241352	41624722	6.2G	0.02	96.14	91.18	52.74
CD3_E	55995352	52713312	7.9G	0.02	96.51	91.95	52.20
IL23_A	59237786	56551580	8.5G	0.02	96.67	92.02	52.77
IL23_B	67655028	63804448	9.6G	0.02	96.59	91.92	52.54
IL23_C	61544716	57684048	8.7G	0.02	96.49	91.77	53.39
IL23_D	53585374	50101108	7.5G	0.02	96.30	91.52	53.13
IL23_E	52941342	49494766	7.4G	0.02	96.23	91.37	52.16
IL23CD3A	55554144	53069836	8G	0.02	96.73	92.11	52.31
IL23CD3B	48185856	44918672	6.7G	0.02	96.21	91.33	52.51
IL23CD3C	51251842	48125674	7.2G	0.02	96.32	91.43	52.61
IL23CD3D	54487030	51175036	7.7G	0.02	96.30	91.45	53.20
IL23CD3E	63578796	59420416	8.9G	0.02	96.66	92.20	53.01

Detail statistics of sequencing data:

(1) Sample name: the names of samples
(2) Raw Reads: the original sequencing reads counts
(3) Clean Reads: number of reads after filtering
(4) Clean Bases: clean reads number multiply read length, saved in G unit
(5) Error Rate: average sequencing error rate, which is calculated by Qphred=-10log10(e)
(6) Q20: percentages of bases whose correct base recognition rates are greater than 99% in total bases
(7) Q30: percentages of bases whose correct base recognition rates are greater than 99.9% in total bases
(8) GC content: percentages of G and C in total bases

Figure 3.10: a summary of the 20 samples sent for RNA sequencing quality experiments including number of reads, clean reads, error rate, Q20 and Q30 percentage and GC content. Figure created by Novogene

3.10.1 Error rates

Phred scores are an internationally recognised system for classifying quality of reads by determining error rates and was created in the late 1990s as a more accurate method of assessing quality of reads than the previous methods. A table of Phred scores is seen in figure 3.10.1.1.

The higher the Phred score the higher the quality of the data such that a Phred score of >40 is superlative quality and a score of 20 is adequate. In all the RNAseq experiments Phred score was calculated using Illumina CASAVA v1.8 software.

Phred quality score	Probability that the base is called wrong	Accuracy of the base call
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1,000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%

Figure 3.10.1.1. A table demonstrating the Phred score and its underlying interpretation.

A graphical representation can be generated for each sample, and one is shown below in Figure 3.10.1.2. The error rate is expected to be slightly higher at lower base levels and tends to decrease with the size of the read as reagents become scarcer. In the figure below I have chosen the sample IL-23_D to demonstrate this effect in one of our samples.

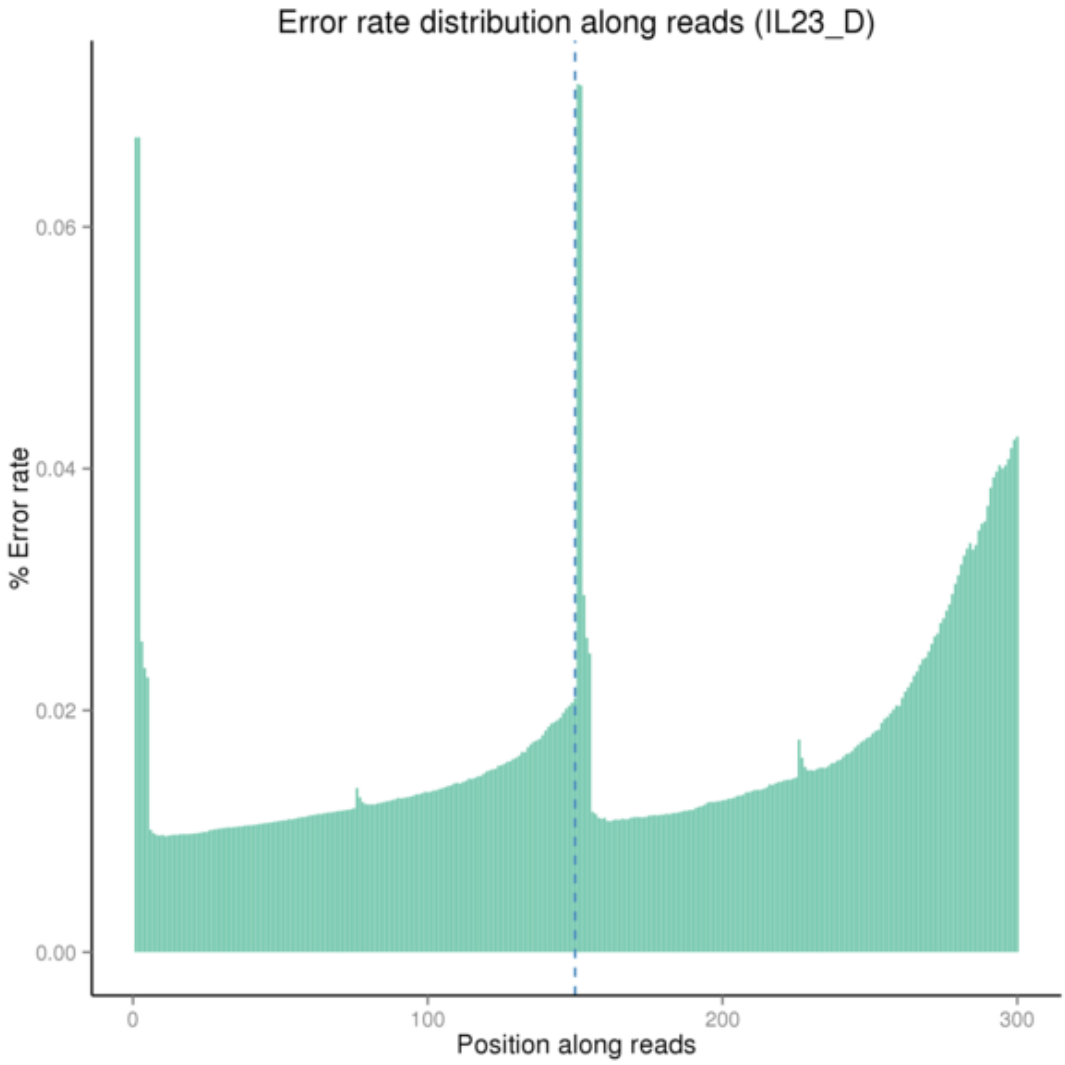


Figure 3.10.1.2 Graph demonstrating error rate associated with position along the read. The dotted line is the end of the read and the area on the right corresponds to the other side of the helix of DNA. Figure created by Novogene

3.10.2. A/T/C/G content

Theoretically A/T and C/G content should be the same but in reality, there will be slight variation. A separation of C/T or A/G ratio may influence expression analysis. There is normally a large error at the small read counts due to variation in the random primers used. An example is given in Figure 3.10.2. showing close alignment of C and G as well as A and T in keeping with expected high-quality data. Similar to Phred scores the absolute value of C/G is not hugely important but the relationship to other samples is key to see if there are any outliers which should be excluded.

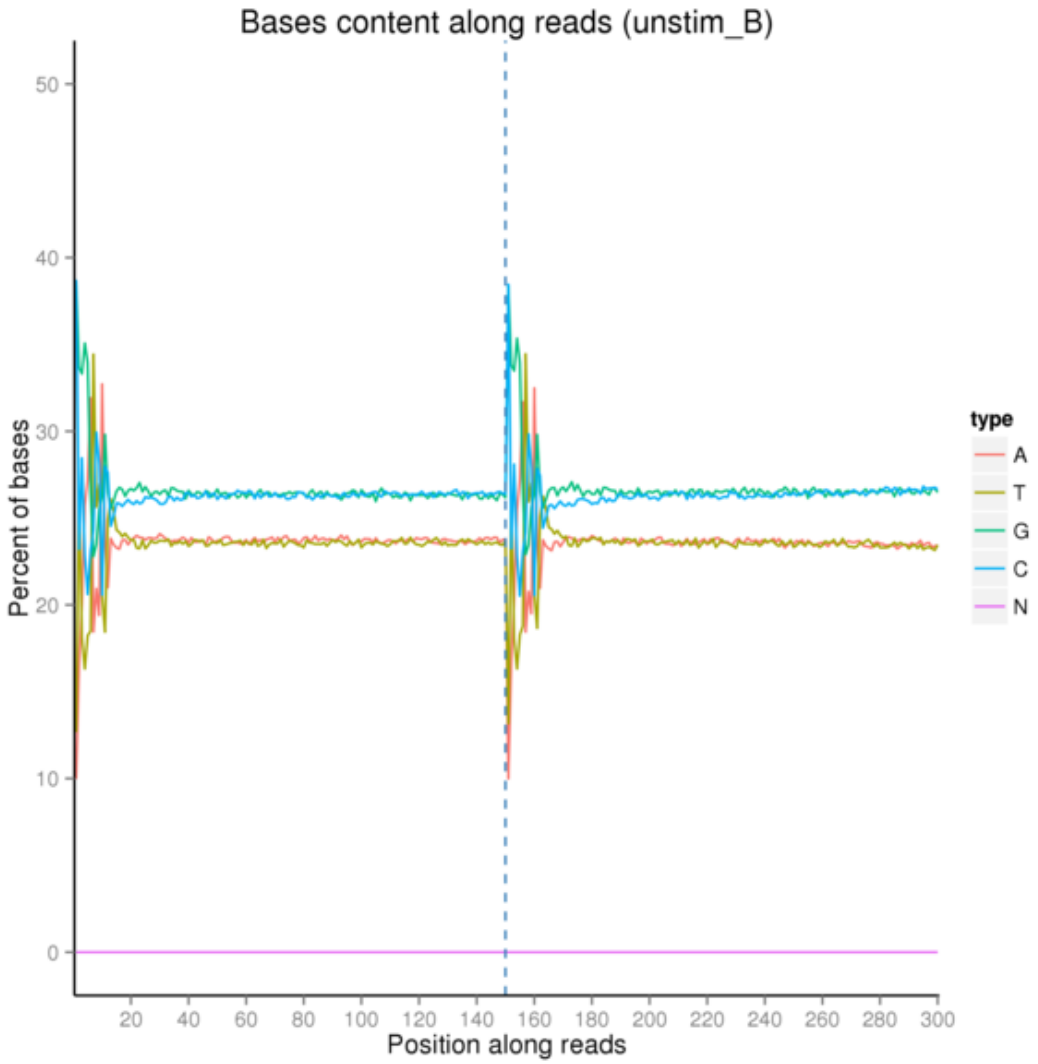


Figure 3.10.2 Showing C, G, A, T quantification in an individual sample (unstim_B). Along the x-axis is the read position with the dotted line separating the forward and backward analysis. The y-axis is the percentage of base pairs each base comprises at that base position. The graph shows good concordance between A/T and C/G bases in this sample. Figure created by Novogene

3.10.3. Data filtering

Raw reads are filtered to remove reads containing adapters or reads of low quality, so that downstream analyses are based on clean reads. Novogene utilized the following approach:

(1) Discard reads with adaptor contamination.
(2) Discard reads when uncertain nucleotides constitute more than 10 percent of either read (N > 10%).
(3) Discard reads when low quality nucleotides (base quality less than 20) constitute more than 50 percent of the read.

An example of the proportion of a single sample and the contents of low quality and adaptor reads is demonstrated in figure 3.10.3. The small proportion of the sample of these constituent parts illustrates the high quality of the sample.

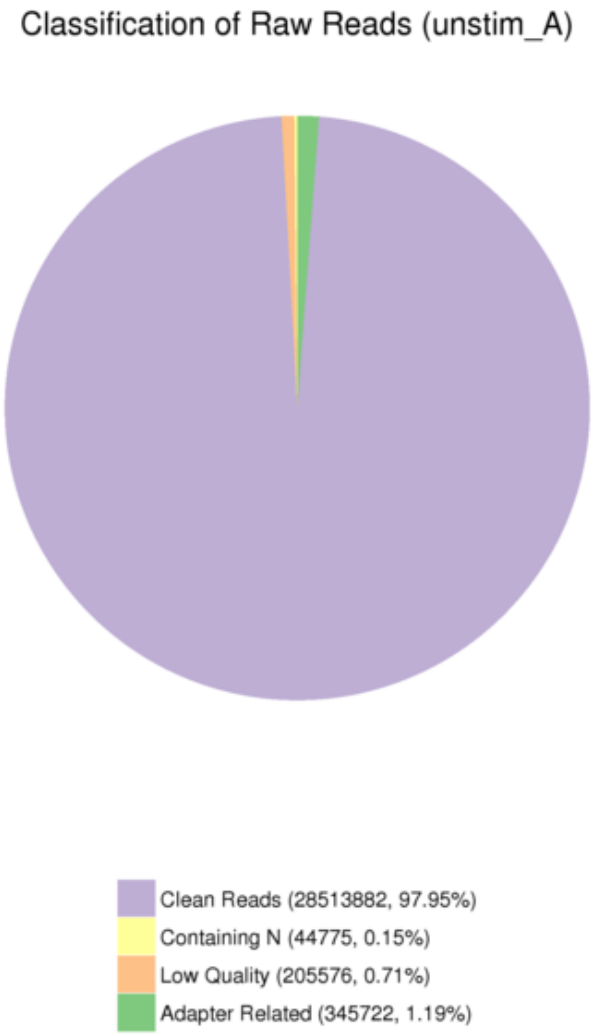


Figure 3.10.3. Pie chart of the breakdown of reads in sample unstim_A of raw reads. The high proportion of clean reads and incredibly low proportion of low quality and adaptor reads implies decent quality RNA. Figure created by Novogene

3.10.4. Alignment

TopHat2 was chosen for mapping to the human genome. The TopHat2 algorithm can be divided into three parts:

(1) Align reads to a reference transcriptome.

(2) Map reads to the exons.

(3) Reads are segmented and then mapped to the adjacent exons.

When the reference genome is appropriate, and the experiment is contamination free,

the Total Mapped Reads or Fragments should be larger than 70% and Multiple Mapped Reads or Fragments should be no more than 10%.

3.11. RNAseq analysis

Aligned read counts produced by Novogene were analysed by the Biomedical Research Centre (BRC) a part of Guy's & St Thomas.' Analysis was performed primarily by Domenico Cozzetto but also by Umar Niazi and Sanjana Sood.

Initial analysis was to perform a layer of QC to look for outliers using the schematic below in figure 3.11. Next the samples were normalised considering cofactors such as lane number, sample ID and treatment.

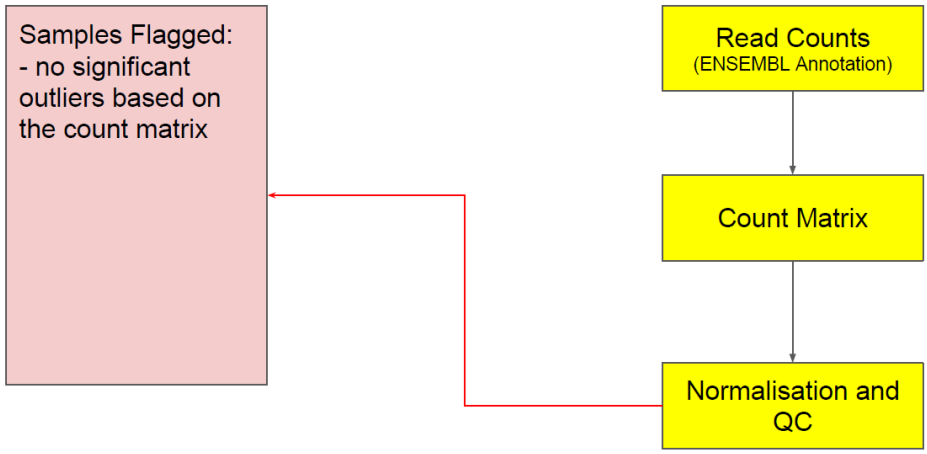


Figure 3.11. Schematic of the workflow utilised by the bioinformaticians to start at aligned read counts then perform quality controls, normalisation, and filtering of read counts such that genes with zero or near zero expression are excluded.

Gene expression level is measured by transcript abundance. The greater the abundance, the higher is the gene expression level. In our RNA-seq analysis, the gene expression level is estimated by counting the reads that map to genes or exons. Read count is not only proportional to the actual gene expression level but is also proportional to the gene length and the sequencing depth. 2 filters are applied before normalization, these are independent of the statistical test being performed. This is done to remove genes with many zeros or no expression.

- **Filter 1:** Genes with average expression of less than 3 across all the samples were removed before performing data normalization.
- **Filter 2:** Remove those genes where most of the counts are zero.

Normalization is done following the strategy suggested by Anders *et al* implemented in the R package DESeq2 (Anders et al. 2013). The statistical modelling for the data is done using a Random Effects Hierarchical model in R and Stan. The Generalized Linear Model assumes a negative binomial distribution for the RNA-Seq data. Differentially expressed genes were then identified using model dependent p-value estimation and False Discovery Rate (FDR) value estimation based on multiple hypothesis testing.

3.12. Statistical definitions

All statistical tests were performed using GraphPad prism 8.2.1. Continuous data are presented as median followed by range, unless stated otherwise. Non-parametric tests were used for comparisons and all p values are two tailed. Principal component analysis and hierarchical clustering was performed using ClustVis (Metsalu and Vilo 2015). Heat maps were created using the MORPHEUS platform provided by the Broad Institute (https://software.broadinstitute.org/Morpheus).

3.13. Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) is a web based curated platform developed by Qiagen to enable interpretation of transcriptomic (and other omic) data. It contains 5 million findings manually curated from the biomedical literature and is constantly updated.

Individual transcripts can provide interesting insights into differentially expressed genes but are limited as they cannot inform a biological process. Identification of up or down regulated pathways and regulators can infer far more meaning to a set of differentially expressed genes and can generate mechanistic hypotheses as well as identify potential therapeutic options.

Experimental data can be uploaded onto the portal including ENSEMBL ID, p-vale, FDR, and fold change data, which is considered during analysis. IPA was used extensively in the analysis of our transcriptomic data especially pathway analysis, upstream and downstream regulators, and associations with diseases & functions.

3.14. Reposited datasets

Reposited datasets are published and free to access datasets, which can be downloaded and interrogated from GEO datasets database. Whole-transcriptome data were downloaded from the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/ geo/). The following publicly available data sets were used in this study: GSE59071 (Vanhove et al. 2015), GSE16879 (Arijs, De Hertogh, et al. 2009), and GSE23597 (Toedter et al. 2011).

GSE59071 (Vanhove et al. 2015) comprised of colonic mucosal biopsies from 97 patients with UC, and 11 controls with normal mucosa from a single centre. Biopsies were taken from the edge of the ulcers in the most inflamed part of the colon (sigmoid or rectum). Disease activity was endoscopically assessed. In UC, there were 74 patients with active disease (endoscopic Mayo subscore 2–3) and 23 with inactive disease (endoscopic Mayo subscore 0–1). All controls

underwent endoscopy for screening of polyps and had an endoscopically normal mucosa.

GSE16879 (Arijs, De Hertogh, et al. 2009) - 24 patients with ulcerative colitis UC refractory to corticosteroids and/or immunosuppression from a single centre. The patients underwent endoscopy with biopsies from diseased colon within a week prior to the first intravenous infusion of 5 mg infliximab per kg body weight. They underwent a second endoscopy with biopsies 4 weeks after the first infliximab infusion in case of a single infusion and at 6 weeks if they received a loading dose of infliximab at weeks 0, 2 and 6. The biopsies were taken at sites of active inflammation but at a distance of ulcerations. Response to infliximab was defined as a complete mucosal healing with a decrease to a Mayo endoscopic subscore of 0 or 1 with a decrease to grade 0 or 1 on the histological score for UC.

GSE23597 (Toedter et al. 2011) - Colonic biopsies were collected at protocol specified time points in the ACT1 study (ClinTrials.gov Identifier NCT00036439) from a subgroup of randomized patients who agreed to biopsy collection. Biopsies were obtained 15 to 20 cm from the anal verge during endoscopies conducted at weeks 0 and 8 of the induction phase. Response was defined as a decrease from baseline in the total Mayo score of at least three points and at

least 30 %, with an accompanying decrease in the subscore for rectal bleeding of at least one point or an absolute subscore for rectal bleeding of 0 or 1

We used these datasets by interrogating the enrichment of our RNA signatures that we generated from the co-culture experiments. Reposited datasets had several advantages in that many were generated from clinical trials (e.g. ACT1 (Arijs, Li, et al. 2009)) and were therefore robustly collected and processed and already had outcome data such as response to infliximab.

3.15. Enrichment score

Having generated transcriptional signatures, we sort to evaluate their ability to separate patient groups. Enrichment scores quantify the amount a pre-defined set of genes is either up or down regulated across the whole genome. This can be repeated for numerous samples and the enrichment scores can be directly compared. Enrichment scores are calculated either through web based or R based platforms of which there are many techniques widely used across the literature including GSEA, ssGSEA, singscore and GSVA each with its own advantages and disadvantages. A review of these different scoring systems is found in a paper by Foroutan *et al (Foroutan et al. 2017)*. We chose to use GSVA due to local experience and

expertise in using this score as well as good correlation of score in the sample numbers we were intending to use i.e., between 25 and 100 samples.

GSVA takes a gene signature and interrogates the abundance of expression of these genes within whole genome RNA sequencing (microarray or RNAseq) which has been obtained either from reposited datasets, datasets generated from our own institution or from industry collaborators. The samples are compared to other samples within the group and an enrichment score is calculated from -1 to +1 to indicate the degree of enrichment of that sample within the group. The enrichment scores are then combined with outcome data to permit comparison of the enrichment sores in the groupings.

3.16 PURSUIT trial

We also used datasets provided by commercial partners. We collaborated with MSD who provided data from the PURSUIT trial of golimumab in UC and comprises of 152 patients all of whom have had a colonic biopsy taken prior to commencing golimumab from the PURSUIT-M phase 3 trial of golimumab versus placebo (Sandborn, Feagan, Marano, Zhang, Strauss, Johanns, Adedokun, Guzzo, Colombel, Reinisch, Gibson, Collins, Järnerot, et al. 2014). All patients

who received golimumab had a colonic biopsy RNAseq performed though we were not given access to RNAseq from patients who received placebo. We provided them with our gene signatures from which MSD calculated the enrichment scores using GSVA. Included with the dataset are clinical features of disease such as: duration of disease, CRP, faecal calprotectin, faecal lactoferrin, total Mayo score, clinical response at week 6, clinical remission at week 6 and mucosal healing at week 6.

3.17 UNIFI trial dataset

We also collaborated with Jansenn to access their UNIFI dataset. The UNIFI trial (NCT02407236) was a large international multi-site placebo controlled double blinded randomised control trial investigating either placebo or 2 ustekinumab dosing regimens. 186 patients received placebo, 180 patients received ustekinumab 130mg and 184 patients received 6mg/kg dose at induction. All patients had a colonic biopsy taken at week 0 from which they performed RNAseq. All patients were reviewed at week 8 endoscopically and clinically. We provided Jansenn with the IL-23 LPMC signature and they calculated enrichment scores using GSVA. They also provided biochemical (CRP, faecal calprotectin and lactoferrin), patient characteristics (disease duration and

total Mayo score) and week 8 endpoints of clinical response, clinical remission, endoscopic remission, and mucosal healing.

4. IL-23 induced transcriptomic changes in lamina propria mononuclear cells from active ulcerative colitis

We postulated that LPMCs, rich with immune cells, would provide the clearest transcriptomic signal as we expected the immune cell compartment to be the site of action of IL-23 and therefore there may be less obfuscation by the transcriptome produced by non-immune cells. However, the generation of LPMCs is technically challenging, provides only a small number of cells with poorer quality RNA.

The full protocol is described in the methods section but in brief, explants or LPMCs were stimulated for 4 hours at 37°C in culture media +/- added soluble IL-23 then homogenised, placed in Qiazol, and placed at -80°C. RNA was then extracted using the RNAeasy kit and then quality and quantity assessments were made. Finally, the optimal samples are chosen for quality, quantity, and clinical phenotype to be sent for RNA sequencing.

4.1 RT-qPCR

Before we embarked on expensive RNAseq analysis we sought to determine if meaningful transcriptomic change had occurred by performing RT-qPCR on the RNA extracted from LPMCs by interrogating the expression of known downstream cytokines and other IBD relevant cytokines, namely IL-22, IL-17A, IFNγ and TNFα. We had aimed to perform RT-qPCR on more gene targets (e.g., IL-10, TGFβ, REG3A, S100A8) but were unable to do so due to due to limited RNA produced.

4.1.1. IL-22 expression is increased when LPMCs are stimulated by IL-23

IL-22 expression by RT-qPCR of LPMCs from 11 UC patients show statistically increased expression compared with LPMCs which were not exposed to any added cytokine see figure 4.1.1.

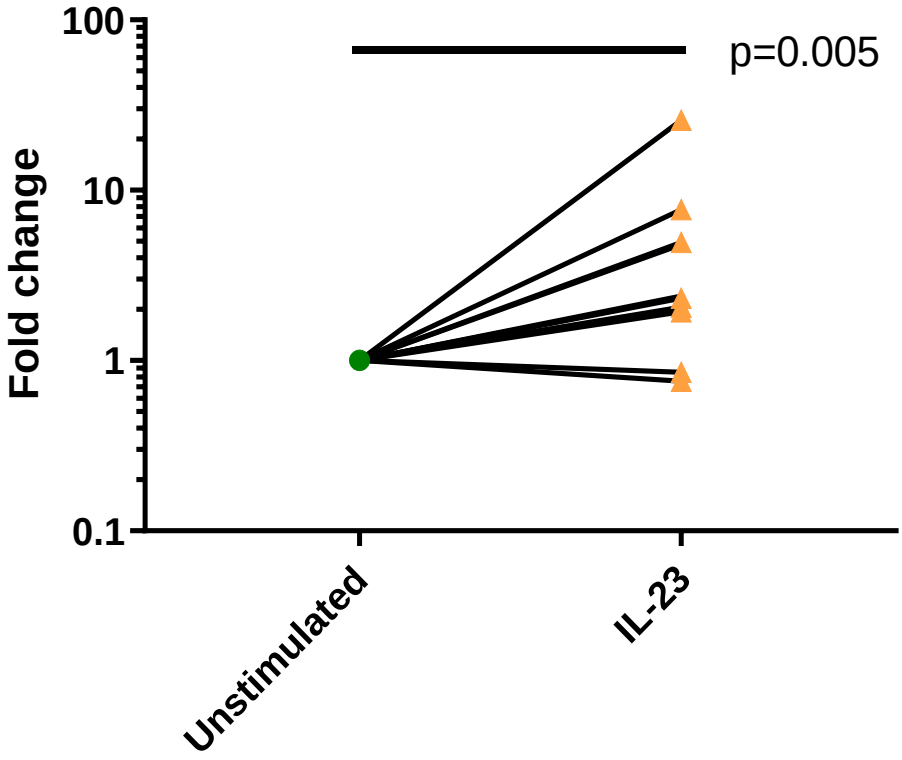


Figure 4.1.1. Graph of IL-22 fold change measure by RT-qPCR in lamina propria mononuclear cells (LPMCs) from patients with active ulcerative colitis cultured with IL-23 or standard media, n=11. IL-23 exposed mean 5.1, IQR 1.9-4.9, SD 7.2). Wilcoxon matched pairs signed rank test, two-tailed p=0.005

4.1.2 IL-17A is not induced in LPMCs when exposed to IL-23 in culture

Surprisingly, we found that IL-17A was not induced in LPMCs exposed to IL-23 as seen in figure 4.1.2. However, as described in the introduction, IL-23 does not directly act on naïve T cells and but acts via specific cytokine signals e.g., TGFβ, IL-1 and IL-6 as well as RORγt. Therefore, this pathway may not be fully activated to produce IL-17A in the 4 hour experimental exposure.

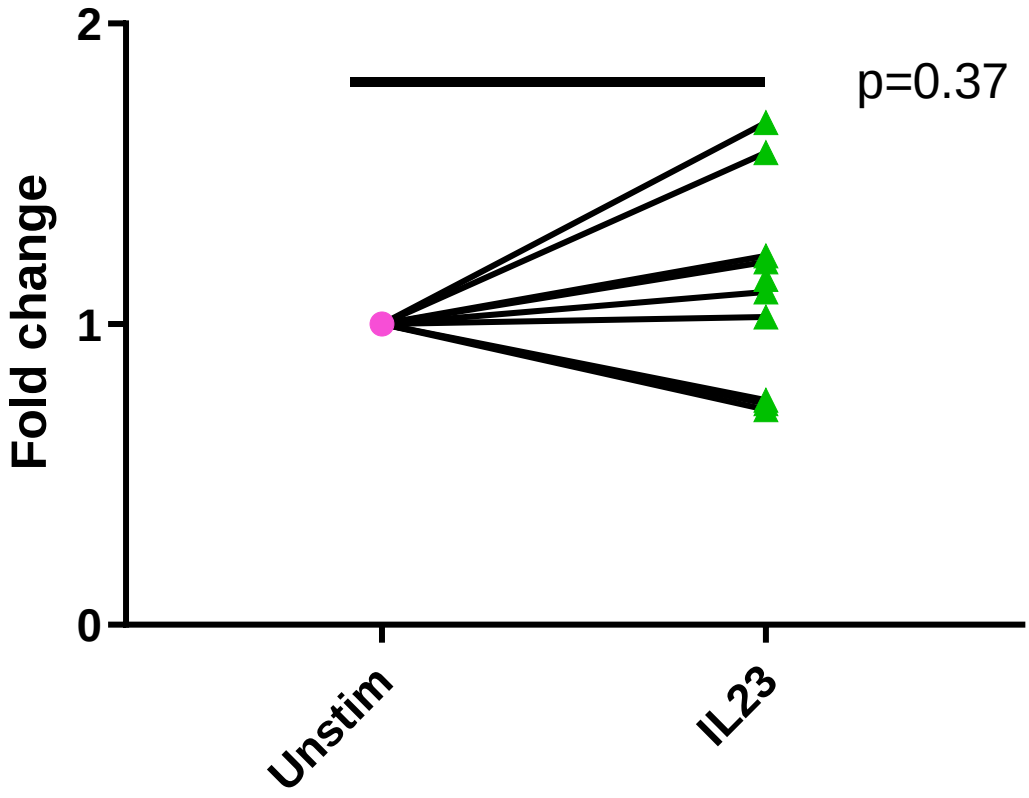


Figure 4.1.2. Graph of IL-17A fold chat. measure by RT-qPCR in lamina propria mononuclear cells (LPMCs) from patients with active ulcerative colitis cultured with IL-23 or standard media, n=10. IL-23 exposed mean 1.1, IQR 0.75-1.31, SD 0.33). Wilcoxon matched pairs signed rank test, two-tailed p=0.37

4.1.3. Interferon-γ is induced in LPMCs when exposed to IL-23

We also investigated IFNγ expression when exposed to IL-23 and found significantly increased expression when compared to the expression of the sample LPMCs with standard culture media as shown in figure 4.1.3 below.

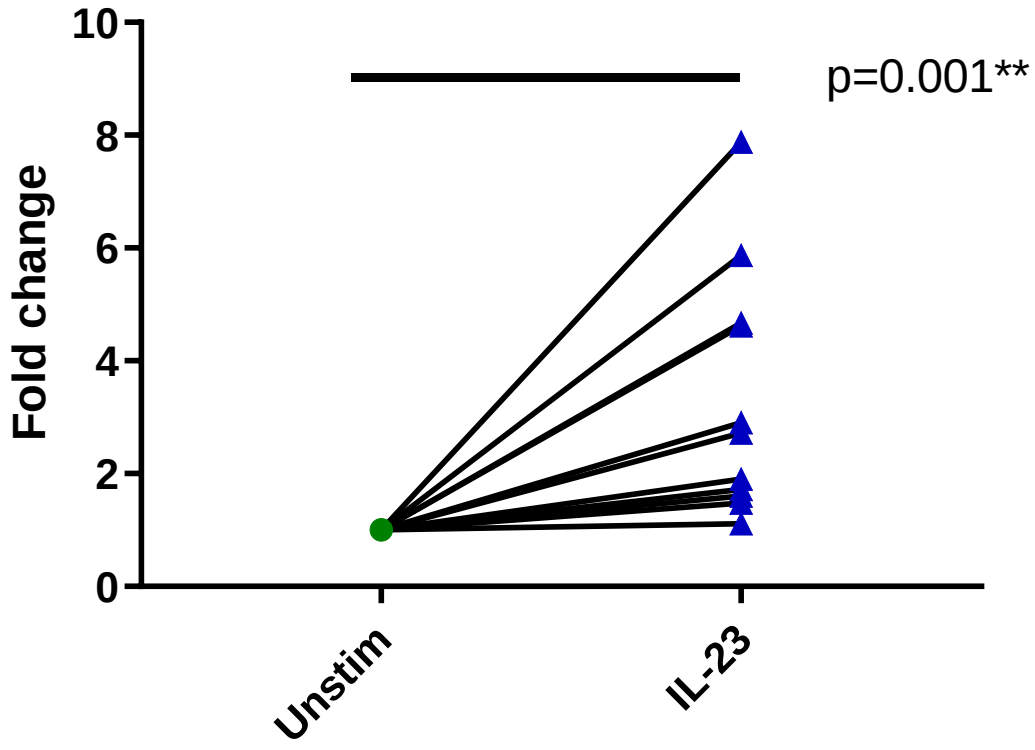


Figure 4.1.3. Graph of IFN-γ fold change measure by RT-qPCR in lamina propria mononuclear cells (LPMCs) from patients with active ulcerative colitis cultured with IL-23 or standard media. n=11. IL-23 exposed mean 3.3, IQR 1.6-4.7, SD 2.2. Wilcoxon matched pairs signed rank test, two-tailed p=0.001

4.1.4 TNF expression was not significantly changed when exposed to IL-23

TNF expression was not significantly changed when exposed to IL-23 ($p=0.73$) which is an expected result as IL-23 is not thought to influence the TNF pathway.

4.2. Sample selection

To achieve the best quality results, we sought to select the samples with the best quality, determined by the RIN score, 260/280 ratio, and quantity determined by Qubit score. A summary of the samples can be found below in table 4.2.1.

Sample ID	Sample Treatment	Collection Date	RNA extraction date	Nanodrop 260/280	Qubit RNA (ng/ul)	Qubit Total RNA (ng/ul)	Bioanalyser RIN score
GST37	Unstim	28-02-17	11-09-17	1.62	12	290	2.4
GST37	IL23	28-02-17	11-09-17	1.70	10	255	2.3
JDB3	Unstim	18-04-17	11-09-17	1.68	Too low	n/a	n/a
JDB3	IL23	18-04-17	11-09-17	1.70	Too low	n/a	n/a
JDB5	Unstim	09-05-17	11-09-17	1.68	58	1455	7.8
JDB5	IL23	09-05-17	11-09-17	1.74	58	1450	7.8
JDB7	Unstim	11-07-17	08-09-17	1.69	24	600	7.50
JDB7	IL23	11-07-17	08-09-17	1.56	Too low	n/a	n/a
JDB8	Unstim	11-07-17	08-09-17	1.67	Too low	n/a	n/a
JDB8	IL23	11-07-17	08-09-17	1.57	Too low	n/a	n/a
JDB9	Unstim	18-07-17	08-09-17	1.66	54	1360	7.60
JDB9	IL23	18-07-17	08-09-17	1.76	84	2105	7.90
JDB10	Unstim	20-07-17	08-09-17	1.78	17	430	n/a
JDB10	IL23	20-07-17	08-09-17	1.78	Too low	n/a	n/a
JDB12	Unstim	08-08-17	11-09-17	1.79	19	475	5.8
JDB12	IL23	08-08-17	11-09-17	1.82	31	775	6.3
JDB16	Unstim	17-10-17	14-11-17	1.70	15	385	5.60
JDB16	IL23	17-10-17	14-11-17	1.36	12	300	Too low
JDB17	Unstim	24-10-17	14-11-17	1.74	25	630	6.90
JDB17	IL23	24-10-17	14-11-17	1.66	28	695	6.10
JDB18	Unstim	24-10-17	14-11-17	2.07	17	415	5.50
JDB18	IL23	24-10-17	14-11-17	1.61	20	505	6.00
JDB22	Unstim	23-01-18	26-01-18	1.65	36	905	7.9
JDB22	IL23	23-01-18	26-01-18	1.76	52	1290	7.6
SP4	Unstim	19-06-17	16-08-17	1.62	37	915	Too low
SP4	IL23	19-06-17	16-08-17	1.62	52	1290	Too low
UC12	Unstim	23-01-18	26-01-18	1.77	93	2325	8.4
UC12	IL23	23-01-18	26-01-18	1.88	93	2330	8.7

Figure 4.2.1. Table of RNA quality data from lamina propria mononuclear cells from patients with ulcerative colitis cultured with IL-23 added or standard media. Quality data includes 260/280 ration, quantity by Qubit, RIN score as well as sample date collected, and RNA extracted. Samples surrounded in a red box were the samples taken forward to send to Novogene for RNAseq (n=6 biological replicates)

The demographics of the patients from whom we took samples is tabulated in Figure 4.2.2. below. We recognised that the characteristic which will dominate transcriptomic change would be the degree of inflammation therefore we only analysed samples with an endoscopic Mayo score of 2 or 3 (the most severe inflammation). Ideally, we would have chosen patients with similar ages, drug profiles and length of disease though given the significant logistical challenges this proved impossible. Whilst there is an age range of 24-54, they are all adults and none of them are over 60. Furthermore, none are on biologics and which we felt was important as they would highly likely alter the transcriptomic prolife and may alter the effect of the IL-23 when added to the media during the experiment. We recognise that thiopurines would also affect the immune pathways but perhaps in less targeted and specific ways compared to biologics.

Sample code	Date of sample collection	Age	Gender	Disease extent 1= proctitis, 2= left sided, 3= extensive	Endoscopic Mayo score	SCCAI	CRP	Faecal calprotectin	Oral 5ASA 0=no, 1=yes	Thiopurines 0=no, 1=yes	Steroids 0=no, 1=yes	Biologics 0 = no 1 = yes	Previous biologic 0 = no 1 = yes
JDB5	09-05-17	54	M	2	2	5	7	not done	0	1	0	0	0
JDB9	18-07-17	27	M	1	2	2	3	not done	0	0	0	0	0
JDB12	08-08-17	36	M	2	3	2	7	302	1	1	1	0	0
JDB18	24-10-17	24	M	2	3	7	3	493	1	1	1	0	0
JDB22	23-01-18	27	F	1	2	2	not done	not done	0	0	0	0	0
UC12	23-01-18	43	F	1	2	5	1	387	1	0	0	0	0

Figure 4.2.2. Table of demographics of the patients with ulcerative colitis whose lamina propria mononuclear cell samples were sent for RNA sequencing

4.3. Quality control analysis

RNAseq was performed by a commercial company called Novogene. We were supplied the raw aligned transcriptomic files and analysis was performed by Umar Niazi in the Biomedical Research Centre at Guy's & St Thomas' Hospital.

Initial analysis was quality control to evaluate for sample outliers.

4.3.1. Principle component analysis demonstrates clustering of samples by sample ID

A principal component analysis (PCA) allows variance between samples to be viewed. This allows for identification of outlier samples as well as looking for covariates which are important for downstream normalisation. Figure 4.3.1.1 and figure 4.3.1.2. show PCAs of 6 samples of LPMCs with biological replicates featuring a sample with standard media (unstimulated) and a sample with media with added IL-23. In figure 4.3.1.1. the samples have been labelled in colour by sample ID whereas in figure 4.3.1.2. the samples are labelled by the treatment to which the samples were exposed.

The most striking feature is that samples are most instructed by the patient ID rather than by the condition they were exposed to. The significant overlap of the ellipses in figure 4.3.1.2. imply significant similarity in the samples themselves and that the exposure to IL-23 has not had a substantial impact on their transcriptome. This is a common finding in RNAseq experiments and is a reassuring feature as the vast majority of the gene expression is driven by the host factors and not just by the exposure of a single immune cue, such as a cytokine to which it is exposed.

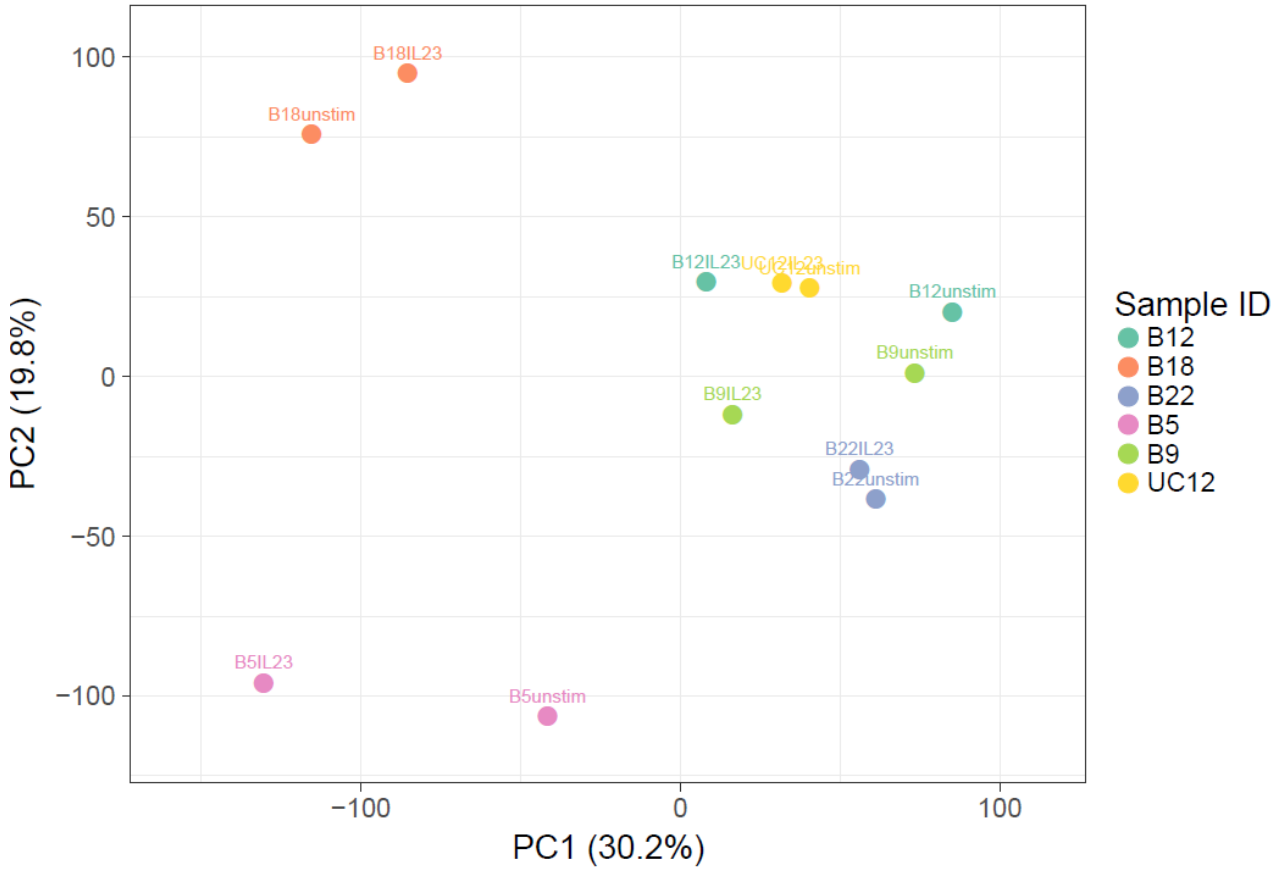


Figure 4.3.1.1. Principle component analysis of RNA sequencing performed on lamina propria mononuclear cells from patients with ulcerative colitis exposed to media with and without IL-23. n=6, colour dots by sample ID.

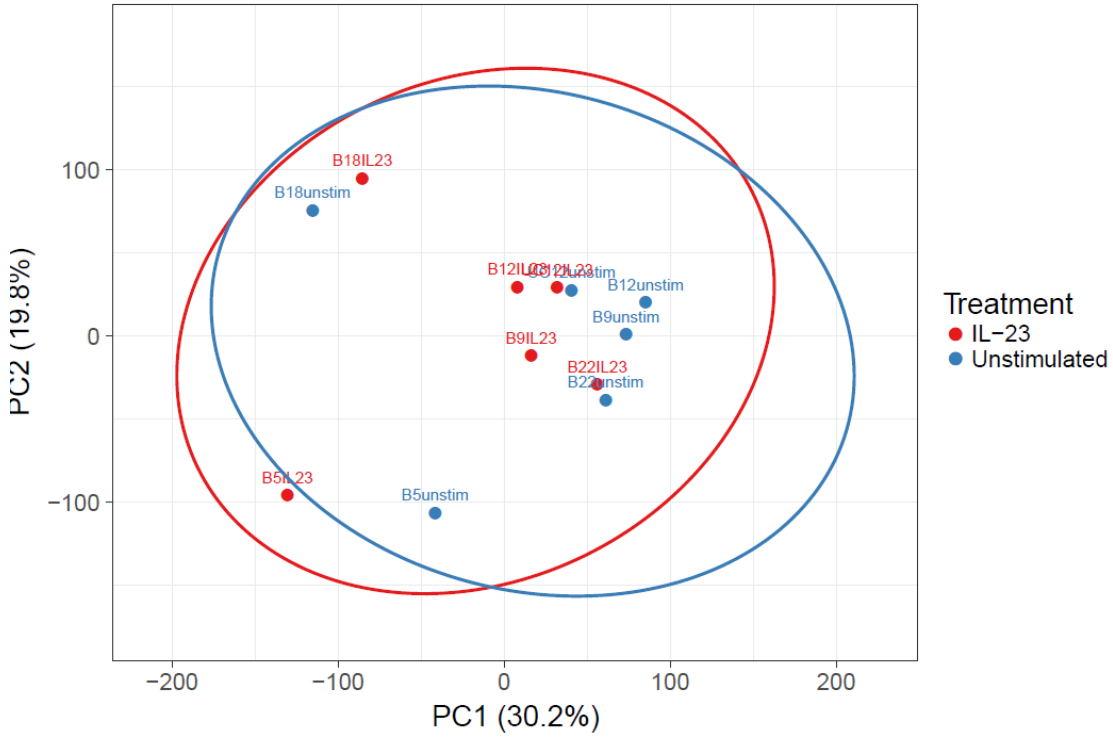


Figure 4.3.1.2. Principle component analysis of RNA sequencing performed on lamina propria mononuclear cells from patients with UC exposed to media with and without IL-23. n=6. Blue dots are samples cultured with standard media (unstimulated) and red dots are samples cultured in the presence of IL-23. Circles represent clustering of the samples with significant overlap with the unstimulated and IL-23 groups.

4.3.2. Hierarchical clustering demonstrates variance by sample ID not condition

An alternative method of analysing the variance amongst samples is to perform hierarchical clustering. In Figure 4.3.2. the colour of the samples is indicated by the sample ID and the matrix clearly demonstrates clustering by this factor in keeping with the sample ID being a key factor in determining the transcriptome.

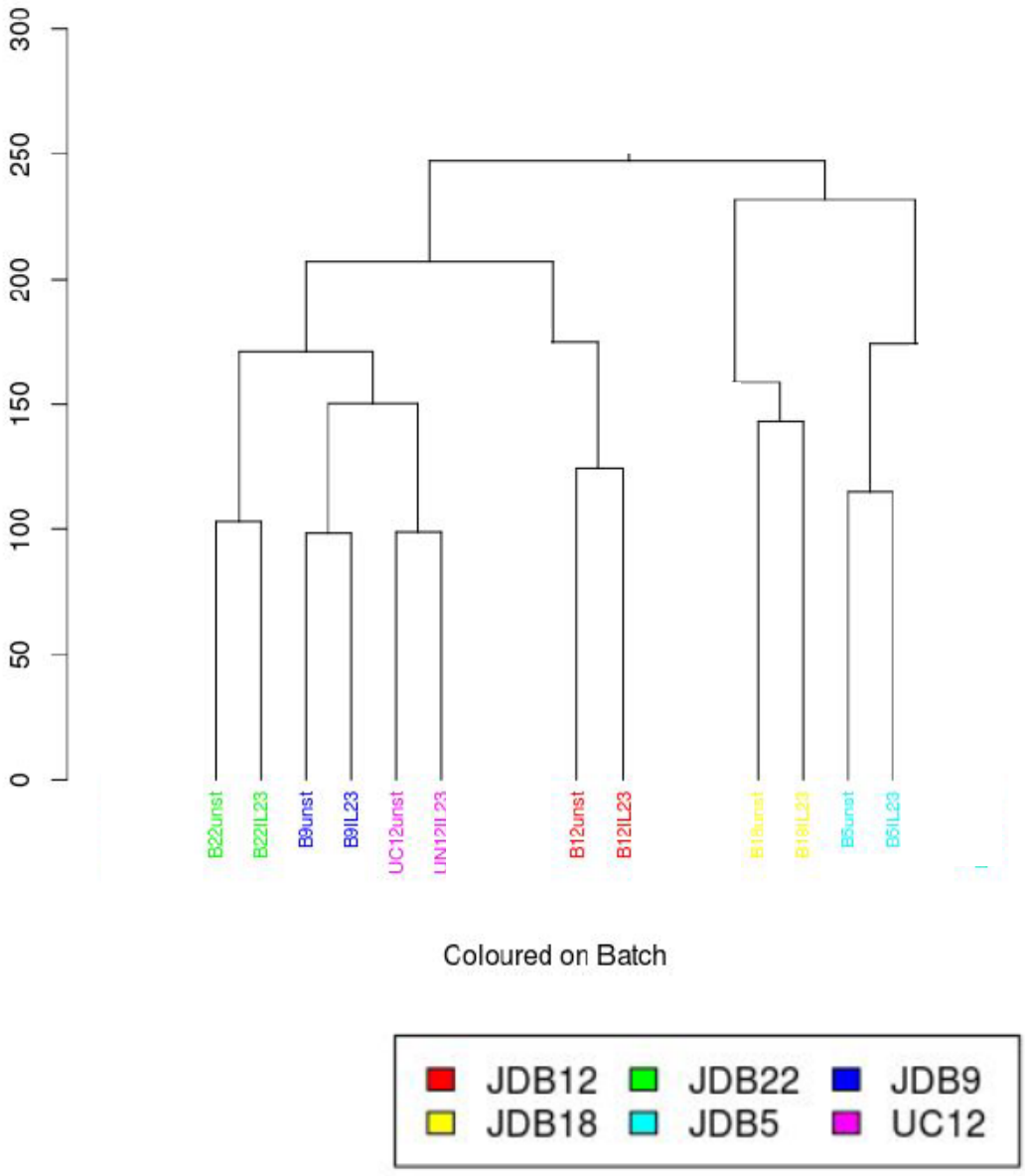


Figure 4.3.2. Hierarchical clustering matrix of lamina propria mononuclear cells from patients with ulcerative colitis (n=6) which have been exposed to culture media with and without IL-23. The colour of the samples is determined by the sample ID. Figure created by Umar Niazi.

4.4. Differentially expressed genes analysis

Differential expressed genes (DEG) analysis was performed by our colleagues at the NIHR Biomedical Research Centre (BRC) at Guy's & St Thomas' namely Dr Sanjana Sood, Dr Umar Niazi, Dr Domenico Cozzetto, and Dr Mansoor Saqi.

A total of 19780 transcripts were identified. Once non-aligned transcripts had been excluded and filtered this number had reduced to 10022.

4.4.1. Volcano plot highlights IL-22 and IFNγ as highly significantly upregulated genes when LPMCs are stimulated with IL-23

A volcano plot was created as seen in figure 4.4.1. This shows a wide spread of transcripts which have been both upregulated and downregulated. The two most significantly expressed upregulated transcripts are IFNγ (fold change 1.78, padj= $8.5x10^{-10}$) and IL-22 (fold change 1.70, padj= $3.3x10^{-8}$). The most upregulated transcript was DOK5 (fold change 5.7, padj= 0.015). The most downregulated transcript was GPR173 (fold change 0.27, padj=0.16).

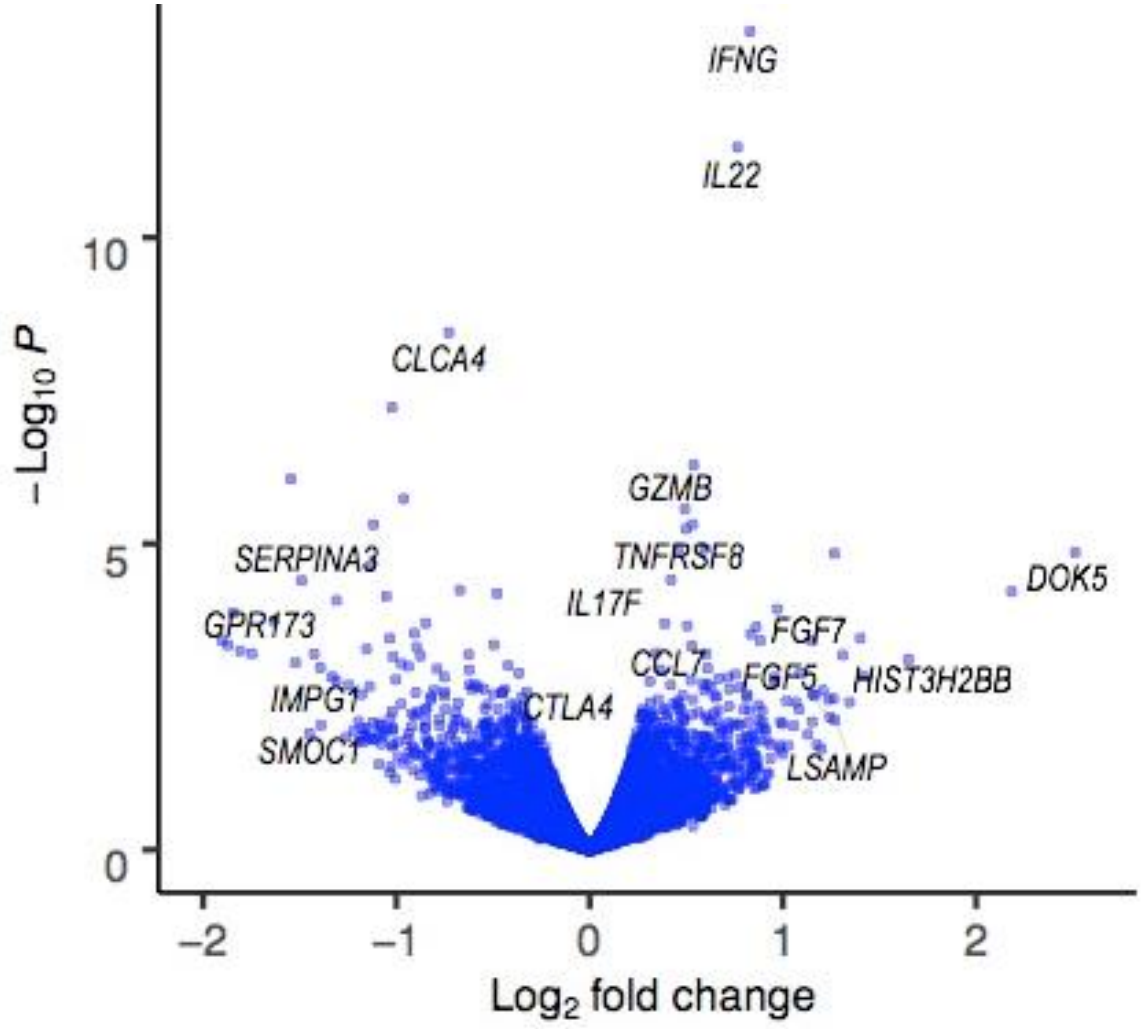


Figure 4.4.1. Volcano plot of IL-23 treated v unstimulated LPMCs from active UC. Y-axis is p value in $-\log^{10}$ and x-axis is the positive and negative fold change expressed as $\log_2$. Figure produced by Polychronis Pavlidis

4.4.2. Heatmaps show widespread effect upon IL-23 treated samples and similarities between biological replicates

Looking at figure 4.4.2.1. shows the top 100 upregulated transcripts for 6 biological replicates of LPMCs from patients with UC treated with either IL-23 enhanced culture media or standard culture media. The heatmap has had hierarchical clustering applied on the y-axis and on the x-axis the samples have been grouped. Broadly the IL-23 treated samples are seen to have a higher expression than the untreated samples but notably there is significant variation between samples. For example, sample ID B5 when examining the unstimulated sample, it already has several transcripts highly upregulated compared to others.

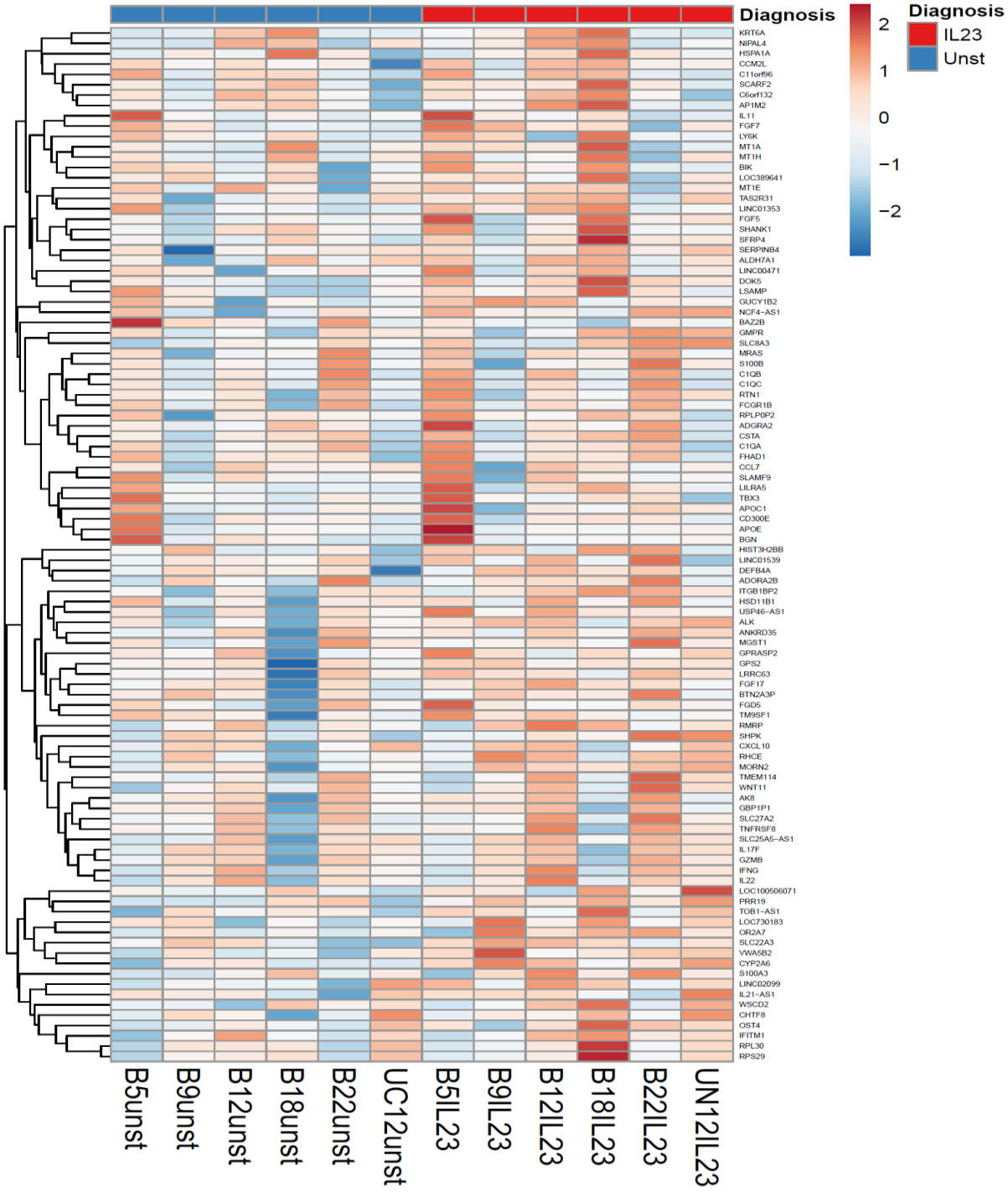


Figure: 4.4.2.1. heatmap of top 100 upregulated transcripts (no significance filter) from differentially expressed genes derived from lamina propria mononuclear cells from patients with active ulcerative colitis have been cultured in the presence v absence of IL-23. n=6. Hierarchical clustering has been performed on the y-axis only.

Examining the data in an alternative way is to perform hierarchical clustering on both the x-axis and y-axis as shown in figure 4.4.2.2. In this figure the top 50 upregulated transcripts have been displayed from the same LPMC IL-23 dataset. This alignment allows direct comparison of biological replicates such that effect of the treatment can be evaluated at a sample by sample level rather than a population level. The most striking observation is that with hierarchical clustering the on the x-axis the biological replicates have clustered together. Whilst not unexpected it is interesting that despite inducing statistically significant transcriptomic changes and analysing the most upregulated transcripts the samples are more alike the original untreated sample than they are to any of the other samples – treated or untreated.

Further observations are that there is heterogeneity of the expression of the transcripts are marked and that some biological replicates appear to have had little transcriptomic response to IL-23, most notably B9, whereas B22 has had a marked response to IL-23 exposure.

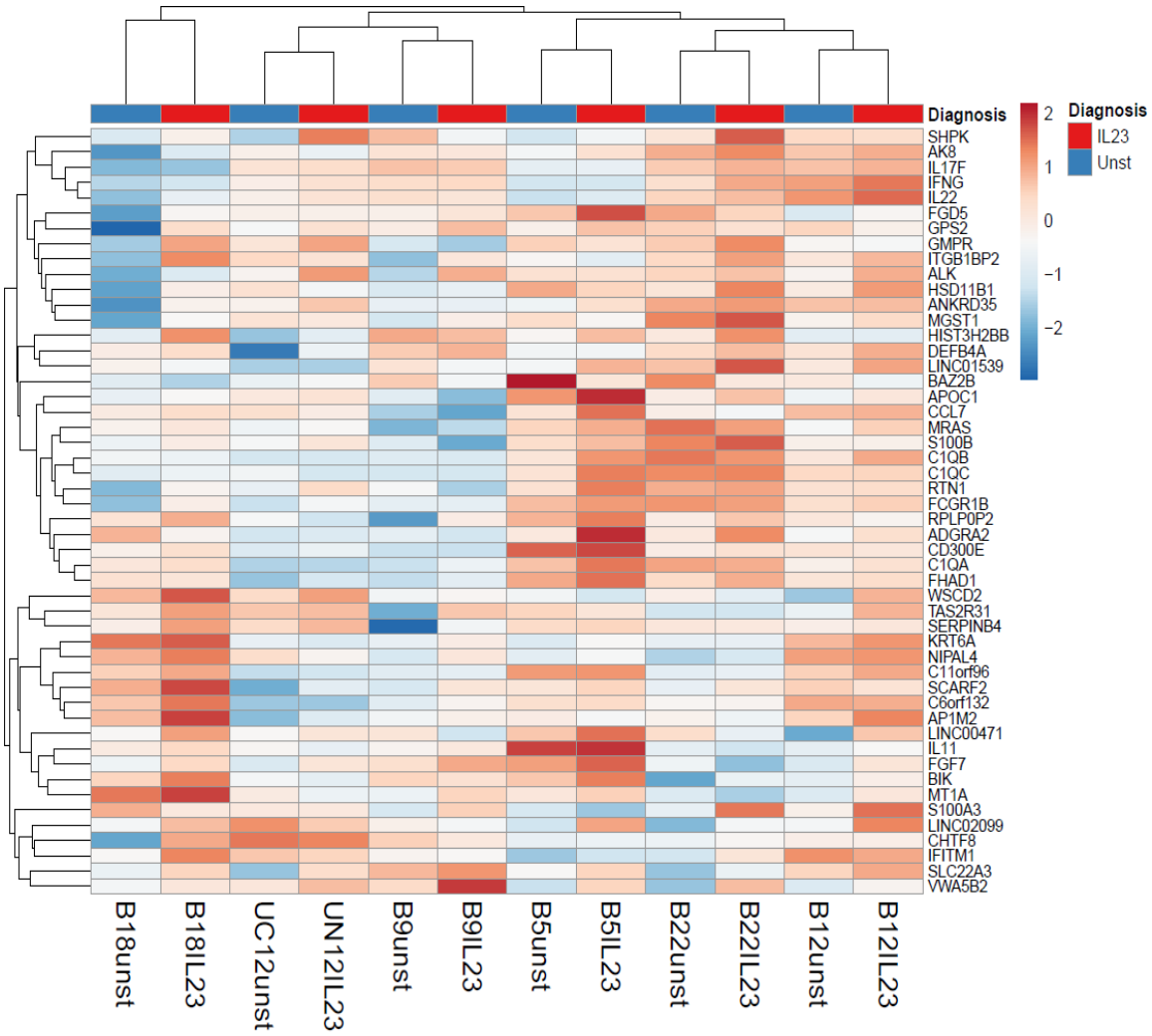


Figure 4.4.2.2. heatmap of top 50 upregulated transcripts (no significance filter) from differentially expressed genes derived from lamina propria mononuclear cells from patients with active ulcerative colitis have been cultured in the presence v absence of IL-23. n=6. Hierarchical clustering performed on x-axis and y-axis.

4.4.3. Filtering transcripts

Different filters can be applied to the dataset depending on the desired statistical parameters. In this dataset we applied a number of different filters on the data summarised below.

4.4.3.1. Padj<0.01 reveals 11 transcripts

A stringent filter of padj<0.01 is commonly used in the literature as a robust means of selecting highly targeted genes. However, in our LPMC experiment this filter only identified 11 transcripts as shown in Figure 4.4.3.1.

ENSEMBL ID	p-value	Padj value	Fold change	Log2 FC	SYMBOL
ENSG00000111537	4.29E-14	8.49E-10	1.778411734	0.830589372	IFNG
ENSG00000127318	3.35E-12	3.31E-08	1.699525542	0.765132044	IL-22
ENSG00000228474	5.19E-07	0.001466767	1.451864484	0.537906799	OST4
ENSG00000130203	4.80E-06	0.007389094	1.448559101	0.534618547	APOE
ENSG00000120949	5.74E-06	0.00811167	1.412156597	0.497900081	TNFRSF8
ENSG00000100453	2.70E-06	0.00485468	1.407605926	0.493243493	GZMB
ENSG00000016602	3.54E-09	2.34E-05	0.603535125	-0.728490358	CLCA4
ENSG00000173258	1.85E-06	0.003662929	0.513571918	-0.961361777	ZNF483
ENSG00000187908	5.91E-08	0.000292046	0.49183552	-1.023752167	DMBT1
ENSG00000164638	4.86E-06	0.007389094	0.460506875	-1.118705399	SLC29A4
ENSG00000168955	8.75E-07	0.001922116	0.342463712	-1.545976969	TM4SF20

Figure 4.4.3.1. Table of differentially expressed genes generated from lamina propria mononuclear cells from patients with active ulcerative colitis are cultured with IL-23 or standard media. The transcripts have been filtered with padj<0.01 revealing only 11 transcripts.

Given the small number of transcripts using this filter the only possible analysis is to look more closely at individual genes. The following genes have a literature base in the field of IBD:

4.4.3.1.1 IL-22 (p=3.4x10-12, padj=3.3x10-8, FC=1.7)

IL-22 is statistically significantly upregulated when LPMCs have been exposed to IL-23 in culture. IL-22 is produced in response to a number of cytokines but chiefly by IL-23 (Powell et al. 2015; Krausgruber et al. 2016; Geremia, Arancibia-Cárcamo, et al. 2011; Powell et al. 2012). Therefore, its upregulation in response to exposure to IL-23 is expected.

4.4.3.1.2 IFNG (p=4.3x10-14, padj=8.5x10-10, FC= 1.8)

IFNγ is statistically significantly upregulated when LPMCs are exposed to IL-23 in culture. IFNγ is canonically produced by CD4 cells when stimulated by IL-12 and is the signature Th-1 cytokine (Monteleone et al. 1997{Parronchi, 1997 #314)}(Ito et al. 2006). However, though that paradigm appears to be changing with a more complex with a number of examples of IFNγ in response to IL-23 which speaks of a Th-1 / Th-17 plasticity (Liu et al. 2011; Ziblat et al. 2018). IL-23 signalling can drive the conversion of Th17 to Th1 cells by shifting the secretion of IL17A to IFNγ in vivo (Hirota et al. 2011).

Moreover, IL-23 may suppress IL-17 expression and enhance IFNγ release through a STAT4/T-bet-dependent pathway, particularly under conditions of decreased TGFβ expression (Ueno et al. 2015). Furthermore, a murine model with CD4+ T cells lacking the IL23R has revealed that IL23R signalling induces colitis, associated with the induction of IFNγ and IL17A co-expressing cells (Ahern et al. 2010).

4.4.3.1.3 GZMB - ($p=2.7x10^{-6}$, $padj=4.9x10^{-3}$, FC= 1.4)

Granzyme B is a cytotoxic serum protease protein that participates in inducing apoptosis of target cells via rapid induction of caspase-dependent apoptosis (Trapani and Sutton 2003; Waterhouse, Sedelies, and Trapani 2006). Granzyme B has been shown to be overexpressed in the LPMCs of patients with IBD compared to healthy controls (Cupi et al. 2014). Furthermore, Granzyme B expression is increased when LPMCs are co-cultured with IL-21 which led to and enhanced ability to induce epithelial cell apoptosis (Cupi et al. 2014). As IL-21 is a downstream cytokine of IL-23 the increase in Granzyme B in our experiment it validated by this paper.

4.4.3.1.4. DMBT1 ($p=5.9x10^{-8}$, $padj=2.9x10^{-4}$, FC= 0.5)

Deleted in malignant brain tumours 1 (*DMBT1*) is a secreted scavenger receptor cysteine-rich protein with predominant expression in the intestine and has been

proposed to exert possible functions in regenerative processes and pathogen defence (Mollenhauer et al. 2000). *DMBT1* expression has been found to correlate with disease activity (Renner et al. 2007) and a deletion allele of *DMBT1* was associated with an increased risk of CD (P = .00056; odds ratio, 1.75) but not for UC (Renner et al. 2007). Furthermore, *DMBT1*$^{-/-}$ knockout mice were found to be more susceptible to DSS induced colitis leading to the hypothesis that DMBT1 is protective in IBD (Renner et al. 2007). This narrative is in line with our experimental model as it would be expected that IL-23 would induce inflammation and so the protective DMBT1 would be downregulated in this situation.

However, another group found that IL-22 induced DMBT1 in colorectal cancer cell line SW403 and that IL-22 and DMBT1 expression correlated in patients with UC (Fukui et al. 2010). These findings are not in line with our experiments as we found upregulated IL-22 expression but downregulated DMBT1. Therefore, further elucidation of the DMBT1 and IL-22 relationship is required before this relationship can be fully characterised.

4.4.3.1.5. Conclusions

IFNγ upregulation in LPMCs in response to IL-23 exposure was an unexpected and highly statistically significant result. However, IFNγ induction by IL-23 has been

described in a number of experimental models and demonstrates the multitude of actions of IL-23 on a number of T cells subtypes. We discuss this further in the conclusions section.

4.4.3.2. Unadjusted p<0.01 filter identifies 222 differentially expressed genes

Utilising a less strict significance filter of unadjusted p<0.01 IL-23 induced differential expression of 222 transcripts (112 upregulated and 110 downregulated), encoding cytokines, chemokines, growth factors, transmembrane receptors, transcription factors, ion channels and enzymes. A diagrammatic illustration of the function of these genes can be seen in figure 4.4.3.2.

The 4 most statistically significantly upregulated transcripts using filter of unadjusted p < 0.01 were: *IFNG* ($P=4x10^{-14}$ FC=1.8), *IL22* ($P=3x10^{-12}$, FC=1.7), *OST4* ($P=5x10^{-7}$, FC=1.5) and *GZMB* ($P=3x10^{-6}$, FC=1.4). Increased expression of other transcripts involved in Th17/ILC3 responses were also significantly upregulated, including *IL17A* ($P=7x10^{-3}$, FC=1.2), *IL17F* ($P=2.x10^{-4}$, FC=1.3) *TNFRSF8* ($P=9x10^{-7}$, FC=1.4), *IL11* ($P=4x10^{-3}$, FC=1.4) and BATF ($P=6x10^{-3}$, FC=1.1).

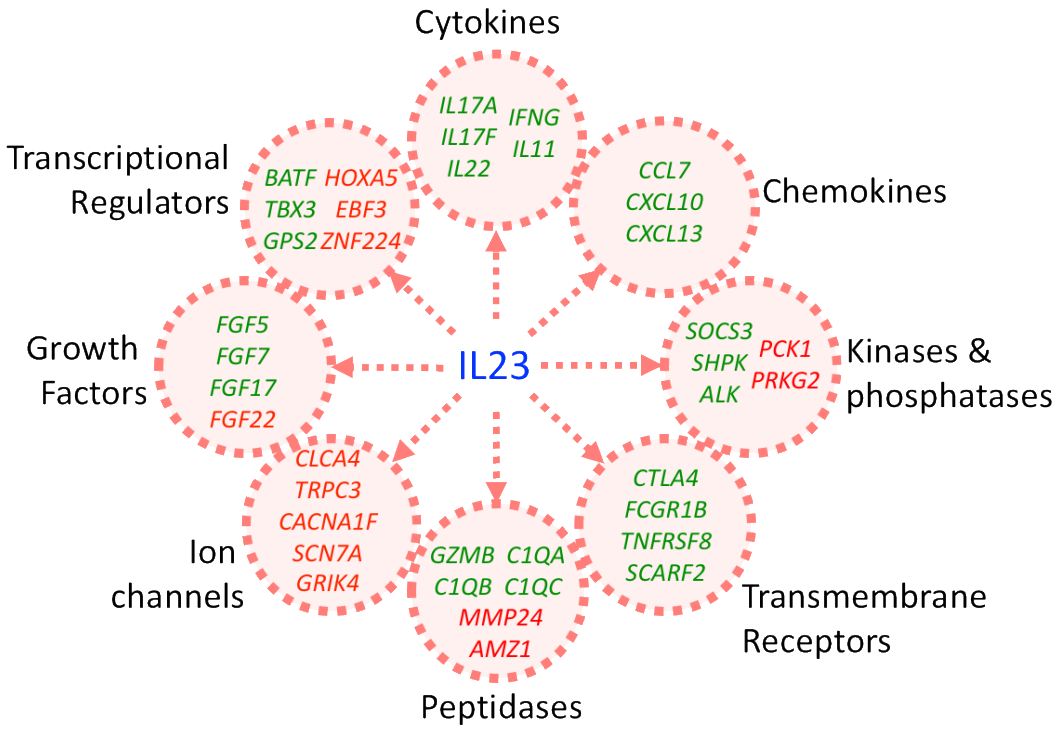


Figure 4.4.3.2. Summary of function of differentially expressed genes of lamina propria mononuclear cells from patients with active ulcerative colitis cultured with IL-23 or standard culture media and filtered by unadjusted $p<0.01$.

4.4.3.2.1. Upstream regulator analysis identifies IL23A and IL12B as well as novel regulators involving IL-1 pathway and toll-like receptor agonists

Upstream Regulator Analysis reassuringly identified the 2 most significantly predicted 2 activators as IL23A ($P=3.5x10^{-7}$) and IL12B ($P=6.6x10^{-7}$), the 2 subunits of IL-23. Although in this experiment we knew the actual upstream regulator (as we had cultured the LPMCs with IL-23) the benefit of this approach is that it would identify mediators likely to share biological activity with IL-23. A summary of the upstream regulators is shown in Figure 4.4.3.2.1. Other predicted activators included IL1 pathway components (IL1A, IL1B, IL1R, MYD88), Toll-like receptor agonists (LPS, imiquimod) and other inflammatory cytokines (IFNG, IL21, IL6, IL33, TNF).

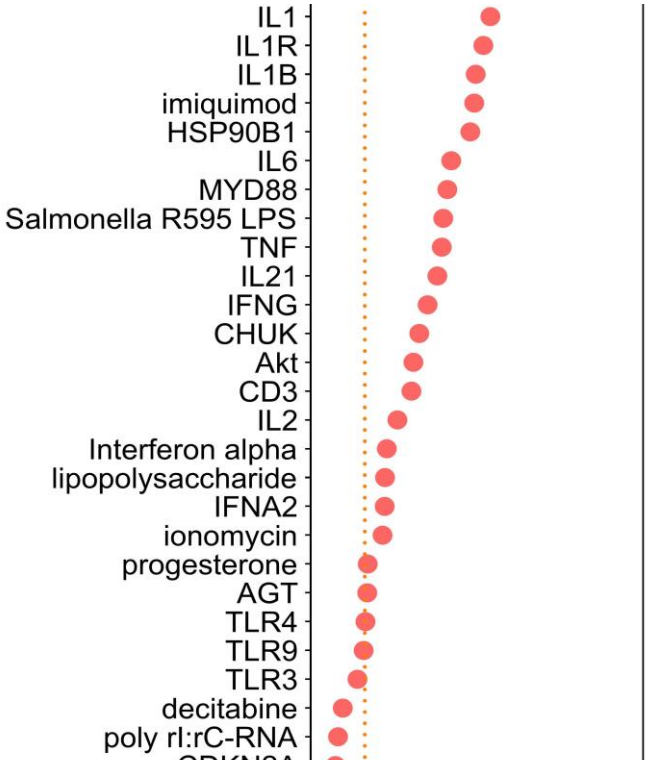


Figure 4.4.3.2.1. Activated upstream regulators identified by Ingenuity Pathway Analysis from differentially expressed genes from lamina propria mononuclear cells

from patients with active ulcerative colitis cultured with addition of IL-23 or standard media.

4.4.3.2.2. Negative predictive regulators

Predicted negative regulators of IL-23 responsive transcriptional changes included recognized anti-inflammatory drugs, such as dexamethasone (P=0.01) and tacrolimus (P=0.006). Dexamethasone use is supported by one study (Sood et al. 2002) though other corticosteroids are more commonly used with a good evidence base (Truelove and Witts 1954; Truelove and Jewell 1974) and the class effect of corticosteroids is likely to be similar. Tacrolimus is effective in inducing clinical remission in UC (Landy et al. 2013; Ogata et al. 2012; Baumgart, Wiedenmann, and Dignass 2003) but the trial numbers are small and most of the literature is based in the pre biologic era and are case control studies. However, several randomised control trials have been performed with another calcineurin inhibitor ciclosporin which has been shown to be effective in UC in well-designed randomised control trials. (Lichtiger et al. 1994; Williams et al. 2016). Furthermore, brodalumab ($P=5.0x10^{-6}$), an anti-IL17 monoclonal antibody was a predicted negative regulator. Interestingly anti-IL17 therapies have been trialled in CD (though not UC) which led to early termination of the trials due to worsening of disease (Targan et al. 2016; Hueber et al. 2012). Other predicted inhibitors included the phosphoinositide 3-kinase inhibitor LY294002, the transcription factor FOXP1 and insulin-induced gene-1 (INSIG1), which are novel potentially druggable targets in IL23-mediated pathology. See figure 4.4.3.2.2. for overview.

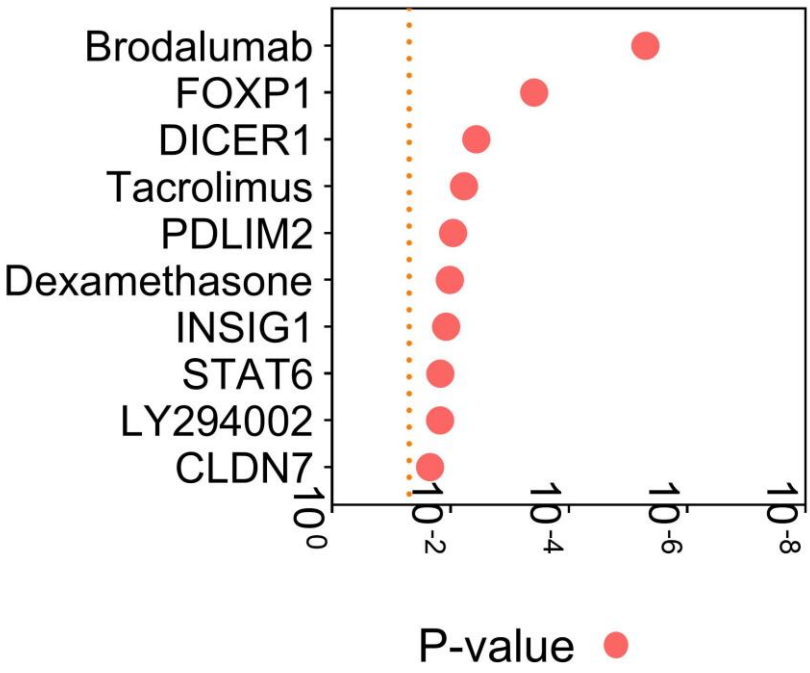


Figure 4.4.3.2.2. Inhibited upstream regulators identified by Ingenuity Pathway Analysis from differentially expressed genes from lamina propria mononuclear cells from patients with active ulcerative colitis cultured with addition of IL-23 or standard media.

4.4.3.2.3. Th17 pathway is significantly enriched by IL-23 stimulation of LPMCs

Next, we investigated which biological pathways were activated by IL-23 using the IPA Canonical pathways instrument. As seen in figure 4.4.3.3.1. the Th17 pathway was the most statistically significantly activated pathway (z-score = 2.646, p= $7.7x10^{-3}$). Interestingly as well as the Th17 pathway there were numerous other pathways associated with IL-17 which have intertwining functions. These results speak to a highly activated Th17 and IL-17 activity when LPMCs are exposed to IL-23.

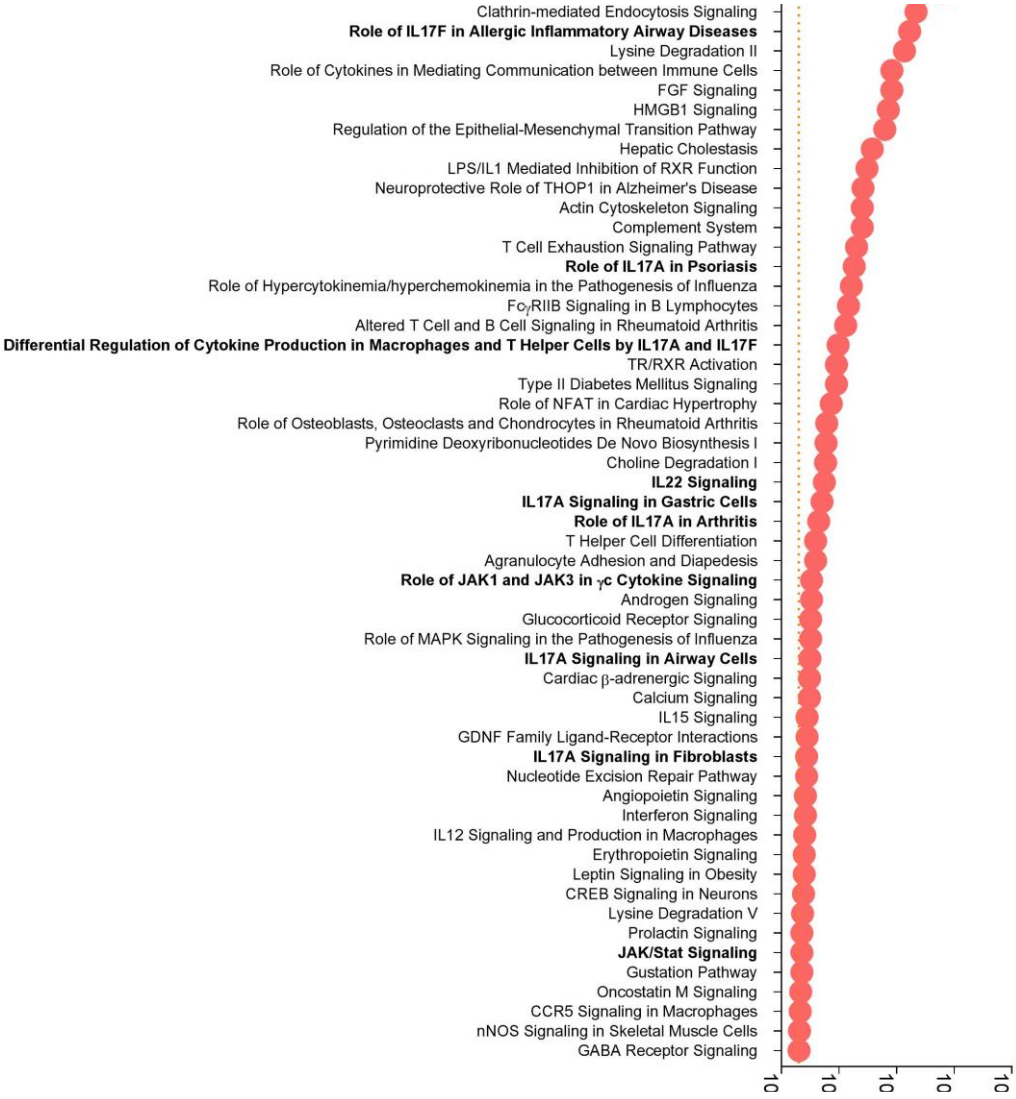


Figure 4.4.3.2.3.1. Summary of activated pathways identified from differentially expressed genes with filter p<0.01 of lamina propria mononuclear cells from patients with ulcerative colitis cultured with IL-23 or standard media. P-value (x-axis, dot, and line). Th17 relevant pathways highlighted in bold

When analysing the transcripts within the Th17 pathway as shown in Figure 4.4.3.3.2 it can be seen the profound and uniformly upregulate effect of IL-23 upon the increased production of these transcripts.

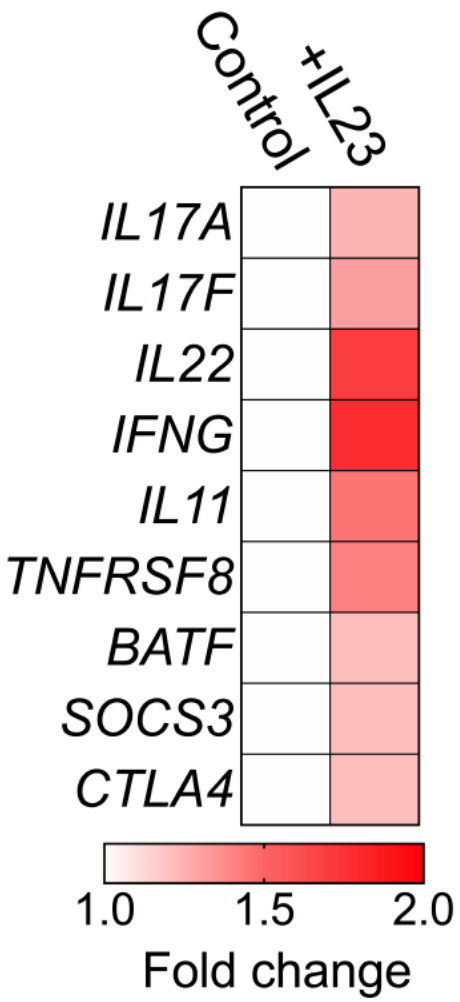


Figure 4.4.3.2.3.2. Comparison of Th17 pathway genes derived from read counts from lamina propria mononuclear cells from patients with active ulcerative colitis stimulated with standard media (control) or IL-23 supplemented media. Fold change calculated by dividing read counts by control value therefore all control values =1

Numerous other pathways as shown in Figure 4.4.3.3.3. were significantly activated including HMGB1 signalling, pathways involved in host recognition of bacteria, and pathways involved in colorectal cancer, angiogenesis (FGF signalling) and T-cell exhaustion.

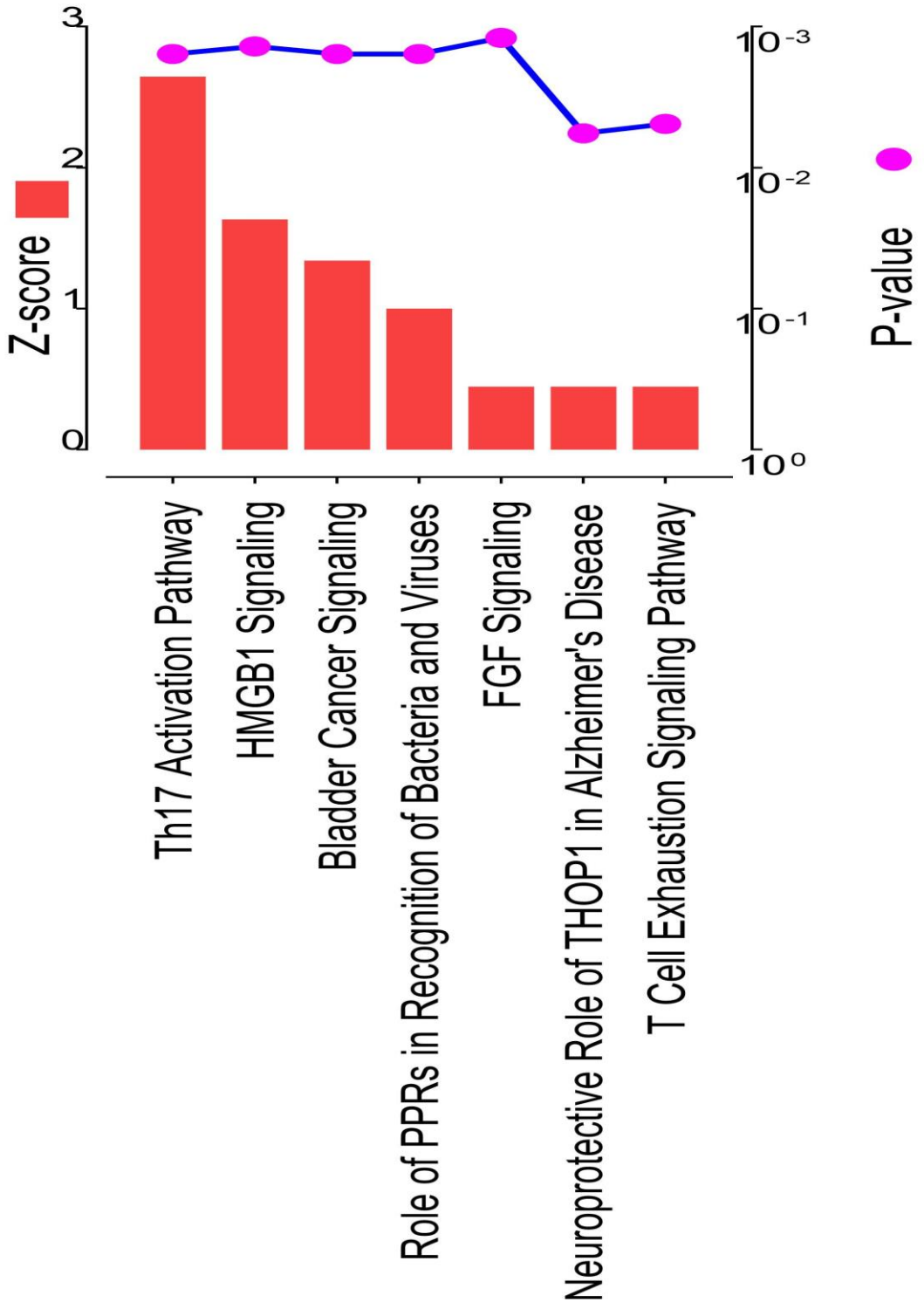


Figure 4.4.3.2.4.3. Graph of activated pathways identified by Ingenuity Pathway Analysis from differentially expressed genes with filter p<0.01 from lamina propria mononuclear cells from patients with active ulcerative colitis cultured with standard media or IL-23 supplemented media

4.4.3.2.4. HMGB1 pathway is pathogenic and a potential therapeutic target for IBD

High motility group box 1 (HMGB1) is an abundant nuclear chromatin-binding protein expressed in almost all cell types (Lotze and Tracey 2005). Once extracellular, HMGB1 can bind to one of several receptors where it behaviors as a proinflammatory cytokine, activating the innate system and mediating a wide range of physiological and pathological responses to exogenous bacterial products or endogenous inflammatory stimuli (Ulloa and Messmer 2006).

In intestinal inflammation, high HMGB1 levels are found in the faeces (Palone et al. 2014)and correlates with endoscopic inflammation and faecal calprotectin (Palone et al. 2014) HMGB1 activates several signaling pathways implicated in the induction of inflammatory cytokines such as IL-6, NF-κB and MAPK (Park et al. 2003; Maeda et al. 2007). Inhibition of HMGB1 appears to be protective in acute DSS colitis (Maeda et al. 2007; Dave et al. 2009).

4.4.3.2.5. IL-23 induces 'cell movement' and 'cell to cell signalling and interaction' of lamina propria mononuclear cells from patients with active UC

Utilising the 'disease & functions' analysis on IPA; IL-23 was predicted to significantly activate (z-score >2) 32 annotated diseases and functions, mapping to 7 high-level categories including "Cell movement" (34.4% of annotations), "Cell-to-Cell Signalling

and Interaction" (28.1%), "Cancer" (15.6%) and "Cellular Development" (12.5%) (see figure 4.4.3.2.4.1.).

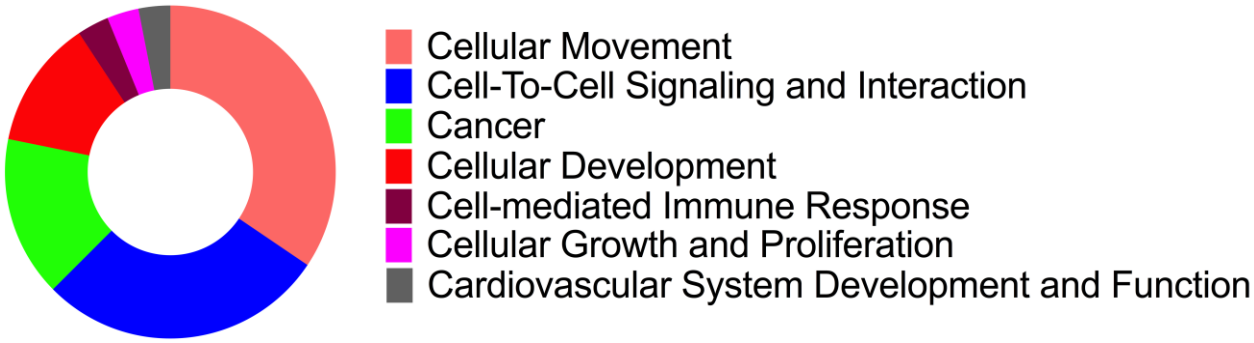


Figure 4.4.3.2.5.1 graph demonstrating the diseases and functions characteristics from ingenuity pathway analysis of differentially expressed genes with filter $p<0.01$ from lamina propria mononuclear cells cultured with standard media and IL-23 supplemented media

Analysis of specific function annotations that were predicted to be activated across all high level categories demonstrated that functions related to cell trafficking and chemotaxis accounted for 20/32 (62.5%) of annotations (figure 4.4.3.2.4.2.). This finding is suggestive of IL-23 inducing gene expression to alter chemotaxis and cell trafficking, functions that have previous been underappreciated and may permit closer attention in further studies as a potential new drug target.

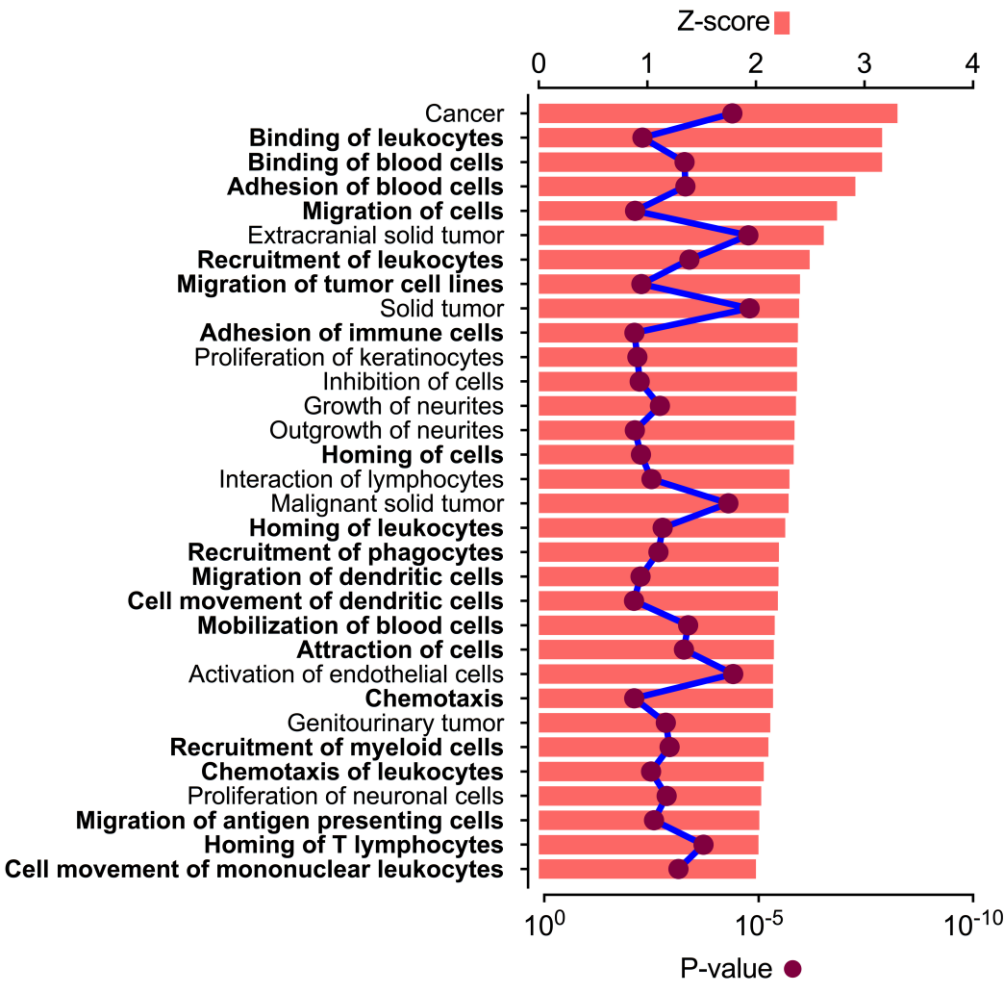


Figure 4.4.3.2.5.2. Graph demonstrating functions characteristics ingenuity pathway analysis of differentially expressed genes with filter of $p<0.01$ from lamina propria mononuclear cells from patients with active ulcerative colitis cultured with standard media and IL-23 supplemented media. Highlighted in bold are the functions involved in cell trafficking and chemotaxis.

4.4.3.3. Conclusions

Taken together these data are consistent with the notion that IL-23 orchestrates a pro-inflammatory transcriptional programme in colonic immune cells from UC patients, and that IL-23 likely shares biological function with the IL-1 cytokine family.

As expected, IL-23 induces the Th17 pathway but induced novel pathways such as HMGB1, cancer and angiogenesis and promotes cell trafficking and chemotaxis, previously unheralded functions. Identification of less well described pathways of HMGB1 and identification of novel negative regulators may lead to more interest in these pathways with potential for new therapeutic strategies blocking these pathways in the future.

A summary of the network analysis of the effect of IL-23 on LPMCs can be seen in Figure 4.4.3.3. which shows the numerous interactions involved. Most strikingly however is that IL-17A, IL-17F, IL-22 and IFNG appear to function as central nodes involved in the coordination of the process leading to the intriguing thought that the interruption of these nodal cytokines may lead to profound interruption in the pro-inflammatory cascade initiated by IL-23.

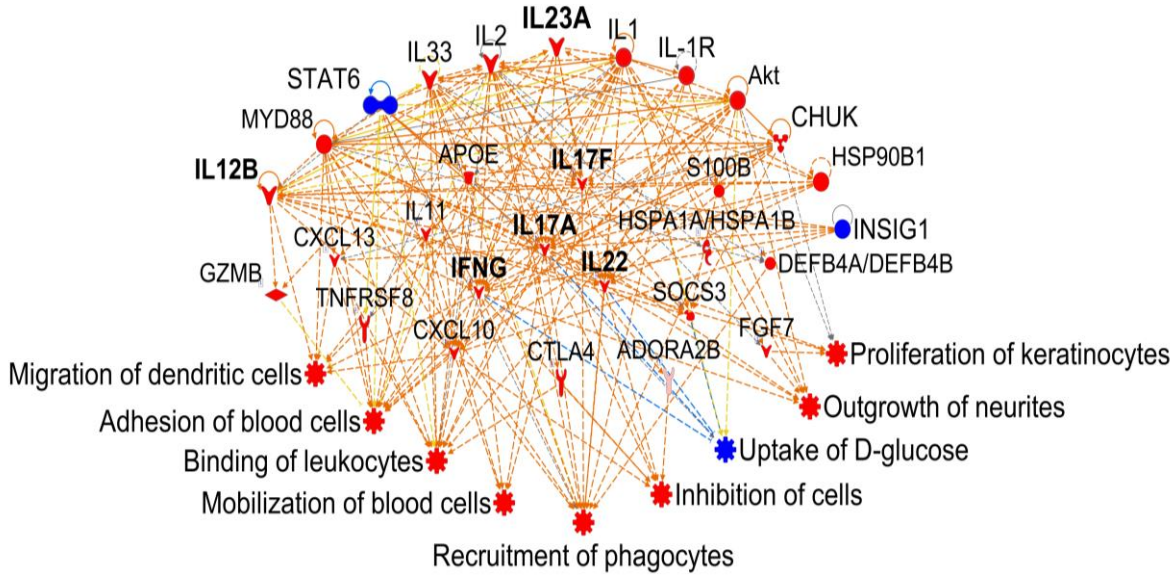


Figure 4.4.3.3. Network analysis of the upstream regulators, top differentially expressed transcripts and functions of differentially expressed genes generated by lamina propria mononuclear cells from patients with active ulcerative colitis cultured with and without IL-23. Red nodes indicate activating regulators and pathways whereas blue indicate inhibiting regulators and pathways. (Figure created by Nick Powell)

5.IL-23 induced transcriptomic changes in explants from active UC

Given the profound effect of IL-23 stimulation upon LPMCs we looked at how IL-23 might impact on cells in tissue, where their cellular communication networks (including their interface with other immune and non-immune cellular players) are preserved.

We therefore chose to use colonic biopsies laced into culture media with and without IL-23. If the relevant pathways were to be preserved in whole colonic biopsies this would provide a less complicated and briefer experimental technique which would necessitate less time and resource to process which would be highly beneficial if it were to be used in clinical practice.

However, there are several differences between LPMCs and whole biopsies. Whilst the number of immune cells may be similar in total number the whole biopsy at the start of the experiment, the LPMCs are expanded in the presence of IL-2 for 48 hours. Therefore, they may be more plentiful in the LPMCs bit also may have more opportunity to degrade and terminate. In whole biopsies, the immune compartment may not be exposed to the IL-23 as it may have to permeate through the biopsy which may limit the effect of the IL-23. Furthermore, other cells, including epithelial cells and

capillaries, will be present in the whole biopsy which are likely to be much less prevalent in the LPMCs, which are likely to have a transcriptomic signal which may be contrary to that of the immune compartment. In conclusion, whilst there are many potentials that there will be significantly differing transcriptomic profiles when investigating the effect of IL-23 on whole biopsies rather than LPMCs due to the above reasons, the potential benefit of the shorter and simpler technique suggests that the experiment was worth pursuing.

We chose to mirror the output that we performed with the LPMCs – namely RT-qPCR and RNAseq to see if the findings could be replicated to those seen in chapter 4.

5.1 RT-qPCR

5.1.1. IL-22 expression is higher by RT-qPCR when colonic explants are co-cultured with IL-23

Using all available samples with sufficient RNA of colonic explants were utilised to evaluate for RT-qPCR which included samples that were subsequently sent for RNAseq as well as those who were not (see 5.1.2 for table of samples). As shown in Figure 5.1.1. there was significantly higher expression of IL-22 when colonic explants were exposed to IL-23. However, we were struck by the heterogeneity of

response across biological samples. This highlights the immunological differences amongst a group of patients with the same disease and that not all patients with UC will have an IL-23 predominant disease. An increase in IL-22 was not unexpected, as it is a canonical downstream cytokine of IL-23, but it was unknown whether exposing colonic explants to soluble IL-23 would induce such transcriptomic change in the time period given.

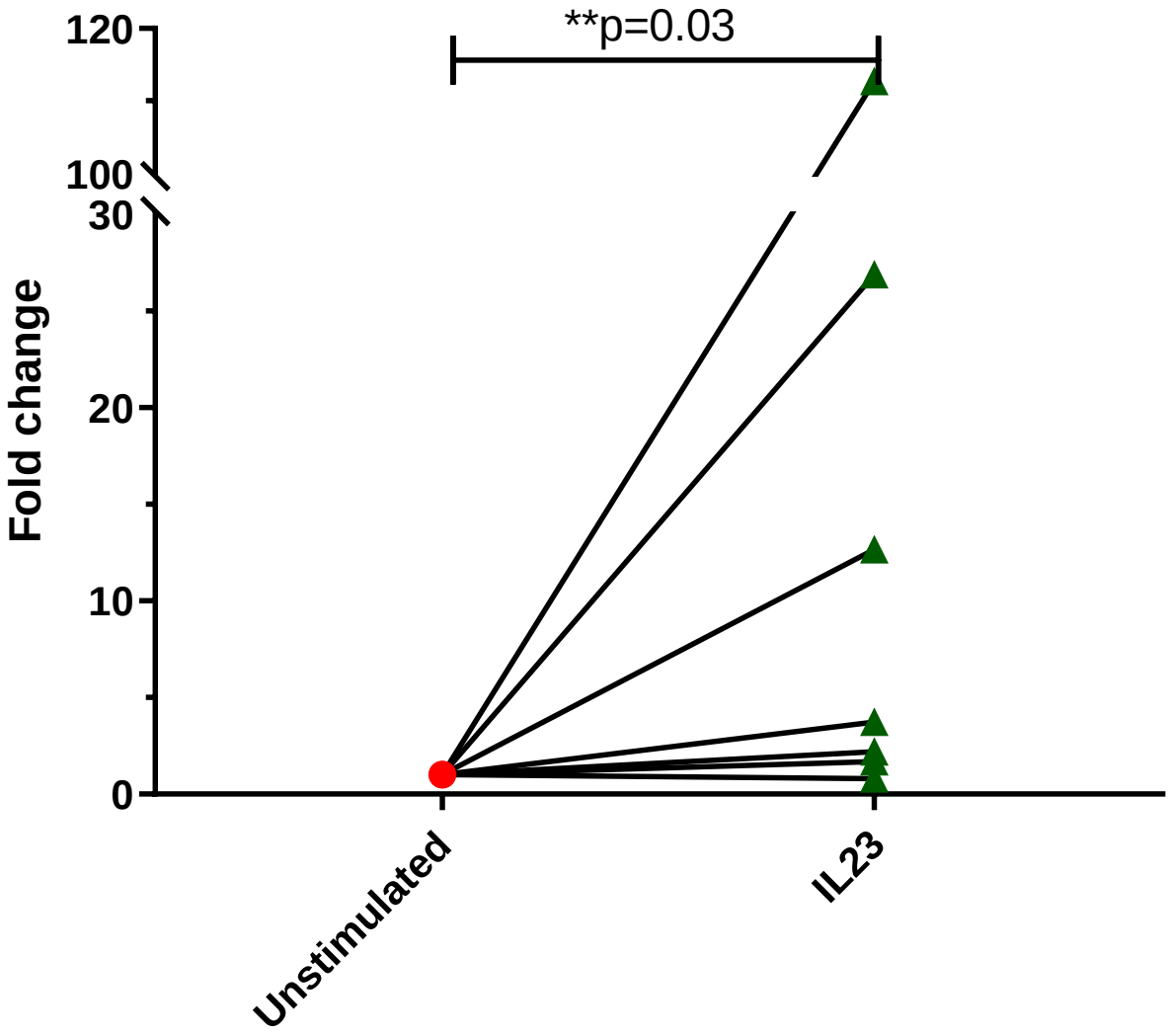


Figure 5.1.1: IL-22 expression by RT-qPCR in colonic explants (n=7) exposed to unstimulated (conventional media) or IL-23 added media. Each sample normalised to the unstimulated expression of the individual sample such that each sample unstimulated = 1. v IL-23 (mean= 23.0, median= 3.7, IQR= 1.7 – 26.9), p= 0.03 using Wilcoxon matched pairs signed rank test.

5.1.2 No increase in IL-17 expression of colonic explants exposed to IL-23

We also measured IL-17, another canonical cytokine to IL-23 but found no difference in expression between the unstimulated and IL-23 exposed colonic explants see figure 4.1.2. below. As IL-17A is a downstream canonical cytokine of IL-23 we had expected to see an increase in expression.

There are technical reasons why IL-17A was not increased such as failure of the primer. Furthermore, it is foreseeable that IL-17A is induced by IL-23 but may be an earlier (or later) induction that has since returned to baseline expression. Given that we stimulated for 4 hours, this seems a likely scenario and is discussed further in 7.1.7.

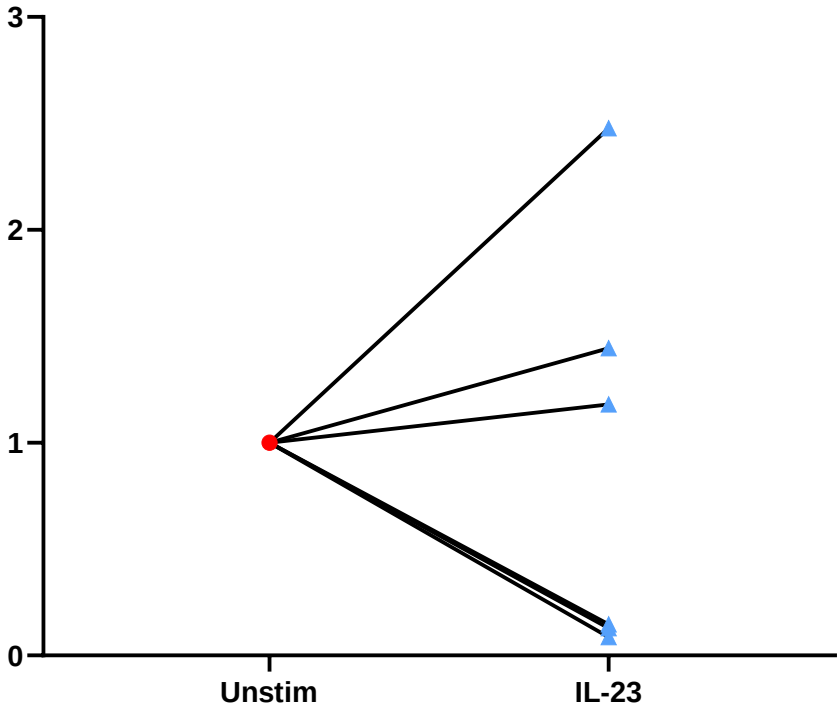


Figure 5.1.2. IL-17 expression by RT-qPCR in colonic explants (n=6) exposed to unstimulated (conventional media) or IL-23 added media. Each sample normalised to the unstimulated expression of the individual sample such that each sample unstimulated = 1. v IL-23 (mean= 0.91, median= 0.66, IQR= 0.12 – 1.70), p= 0.84 using Wilcoxon matched pairs signed rank test.

We also investigated a number of other IBD relevant cytokines such as Interferon-γ , TNF, OSMR, S100A8 but there was no statistical difference in expression between the unstimulated and IL-23 exposed explants (data not displayed).

This set of RT-qPCRs provided a 'mixed bag' of results. Whilst reassuring that IL-22 expression was enhanced by IL-23 it was disappointing that IL-17 as a canonical downstream cytokine of IL-23 did not have statistically significant change in expression. Furthermore, other IBD related cytokines had no differential expression. However, we felt that the IL-22 expression was sufficient evidence that IL-23 was having an effect on the transcriptome of the explants that we decided to move forward to sending the samples for RNAseq.

5.1.2. Sample selection for RNAseq

We aimed to send 5 biological replicates for RNA sequencing from patients with active UC with successful experiments and sufficient decent quality RNA. We aimed to perform the experiments on 10 patients then select the best to take forward for RNAseq.

Once we had performed 9 experiments, we then proceeded to choose the samples with the best quality to move forward to RNAseq. Summary of quantity and quality of the samples is found in figure 4.2 below.

Sample ID	Sample Treatment	Collection Date	RNA isolation date	Nanodrop	Qubit		Bioanalyzer
				260/280	RNA (ng/ul)	Total RNA (ng)	RIN score
GST30	Unstim	07-02-17	25-04-17	1.57	17.80	445	1
GST30	IL23	07-02-17	25-04-17	1.31	Too low	n/a	n/a
GST31	Unstim	07-02-17	25-04-17	2.06	91.20	2280	2.3
GST31	IL23	07-02-17	25-04-17	2.05	99.60	2490	3.8
GST35	Unstim	22/02/2017	02-08-17	2.04	304.00	7600	7.7
GST35	IL23	22/02/2017	02-08-17	2.02	182.00	4550	5
GST37	Unstim	28-02-17	02-08-17	2.05	53.60	1340	6.5
GST37	IL23	28-02-17	02-08-17	2.04	222.00	5550	7.7
GST52	Unstim	28-03-17	16-08-17	1.86	49.40	1235	7.2
GST52	IL23	28-03-17	16-08-17	1.94	47.00	1175	7.7
JDB1	Unstim	11-04-17	17-05-17	1.82	66.8	1670	5.4
JDB1	IL23	11-04-17	17-05-17	2.05	84.8	2120	4.2
JDB2	Unstim	13-04-17	03-08-17	2.02	220	5500	4.9
JDB2	IL23	13-04-17	03-08-17	2.12	784	19600	4.4
JDB3	Unstim	19-04-17	03-08-17	2.09	476	11900	7.2
JDB3	IL23	21-04-17	03-08-17	2.08	432	10800	6
JDB4	Unstim	18-04-17	17-05-17	2.11	49.4	1235	3.9
JDB4	IL23	18-04-17	17-05-17	2.23	50.6	1265	3.2

Figure 5.1.2. Table demonstrating the samples taken for colonic explant experiment with the related RNA quantity and quality. Quantity was measured by Qubit in ηg/μl. All samples were eluted in 25μl, so total RNA calculated as Qubit RNA x 25. 260/280 ratio as quantification of purity of the RNA was calculated by Nanodrop. RIN scores as a reflection of quality of RNA was measured by bioanalyser 2100. Samples chosen to take forward to RNAseq are surrounded in a red box

We chose samples with at least 1000ng of total RNA (>1000ng was ample to perform RNAseq and have some remaining for RT-qPCR) and the ones with best RIN and 260/280 scores. However, the quality of the RNA was not as good as hoped. Ideally Novogene were requesting RIN scores of >6.8 though this was challenging as there was an expected degradation of RNA during the culture phase. We chose to include sample JDB2 even though the RIN score was below 6.8 (RIN scores 4.4. and 4.9) as we wanted to move forward with the LPMC experiments and could not afford more time collecting samples for the explant experiments.

Below in figure 5.2.2. is a table of demographics of the chosen patients. We wanted to make the samples as homogenous as possible by selecting patient with the same characteristics as possible. We felt the most influential factors on the transcriptome would be endoscopic severity and therefore we chose only patients with the same level of severity (i.e., Mayo 2). Further factor would be drugs – particularly biologics so we chose patients without these drugs. We also recognise the influence of age, particularly advanced age, though due to the above restrictions we were inclined to include one patient (JDB3) who is older than initially hoped.

Sample code	Date of sample collection	Age	Gender	Disease extent 1= proctitis, 2= left sided, 3= extensive	Endoscopic Mayo score	SCCAI	CRP	Faecal calprotectin	Oral 5ASA 0=no, 1=yes	Thiopurines 0=no, 1=yes	Steroids 0=no, 1=yes	Biologics 0 = no 1 = yes	Previous biologic 0 = no 1 = yes
GST35	22-02-17	28	F	1	2	4	8	Not done	0	0	0	0	0
GST37	28-02-17	27	M	3	2	7	1	Not done	1	0	0	0	0
GST52	28-03-17	28	M	3	2	2	5	Not done	1	1	1	0	0
JDB2	13-04-17	43	M	3	2	0	1	Not done	0	0	0	0	0
JDB3	18-04-17	78	M	2	2	6	5	319	1	0	0	0	0

Figure 5.1.2. Table of demographics of patients with UC whose samples underwent colonic explant experiment and were taken forward for RNAseq

5.2. Quality control analysis

Differential expressed genes (DEGs) analysis was performed by our colleagues at the NIHR Biomedical Research Centre (BRC) at Guy's & St Thomas' namely Dr Sanjana Sood, Dr Umar Niazi, Dr Domenico Cozzetto, and Dr Mansoor Saqi.

Initial analysis was quality control to evaluate for sample outliers.

5.2.1. Principle component analysis demonstrates clustering of biological samples of colonic explants

A principal component analysis (PCA) allows variance between samples to be viewed as seen in figure 4.3.2. This allows for identification of outlier samples as well as looking for covariates which are important for downstream normalisation. Figure 4.3.2. shows a PCA of colonic explants with 5 biological replicates with unstimulated and IL-23 samples. The most striking feature is that the most similar samples are instructed by the patient ID rather than by the condition they were exposed to. This is a common finding in RNAseq experiments and is a reassuring feature as the vast majority of the gene expression is driven by the host factors and not just by the exposure of a single immune cue, such as a cytokine to which it is exposed.

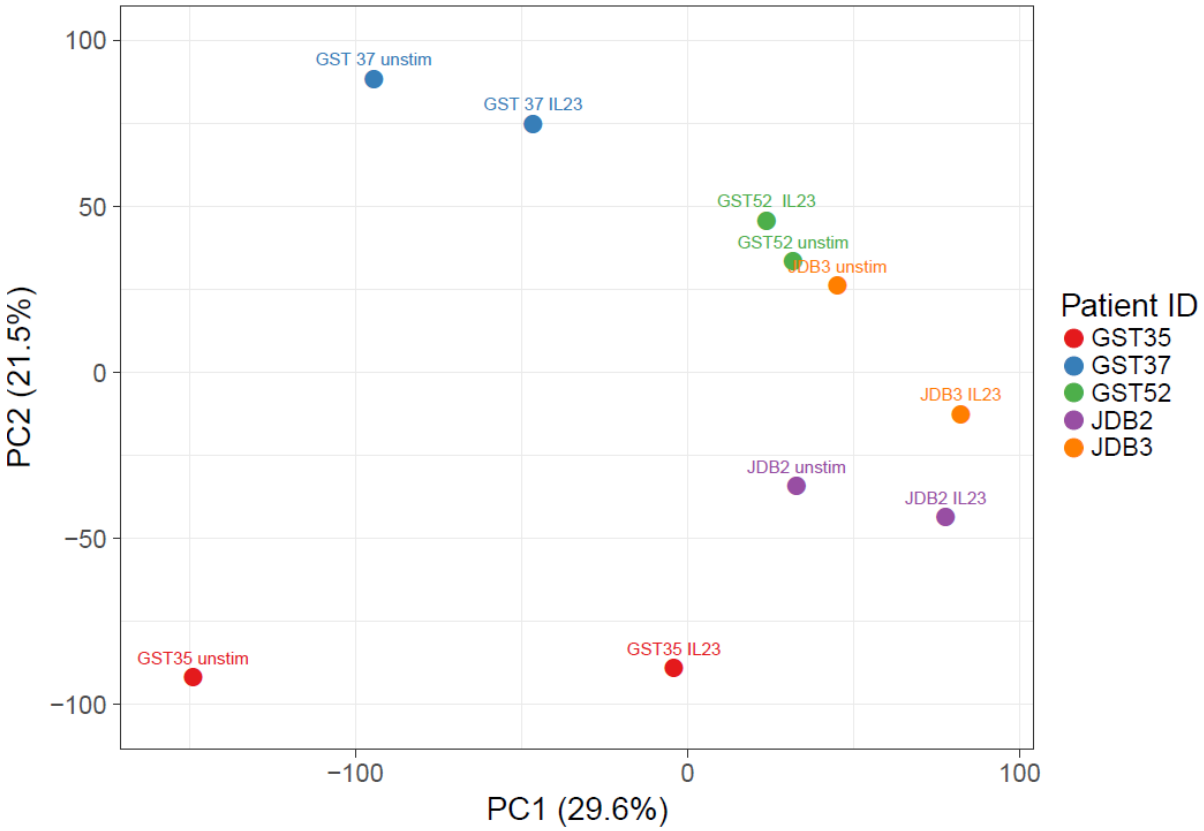


Figure 4.3.2. Principle component analysis of transcriptomic profile of colonic explants from patients with ulcerative colitis cultured in standard media (unstimulated) or with the addition of IL-23. Each patient ID is a represented with distinct colour plots.

5.2.2. Hierarchical clustering demonstrates lane number as covariate

An alternative method of analysing the variance amongst samples is to perform hierarchical clustering. As shown in figure 4.3.3. which shows hierarchical clustering of the colonic explant samples where the colour representation is by lane number used during RNAseq. As can be seen from the figure there are 7 lanes that have been used but lane 7 (purple colour) appears to cluster more than would be expected. Therefore, lane number was included as a covariate during DEG analysis.

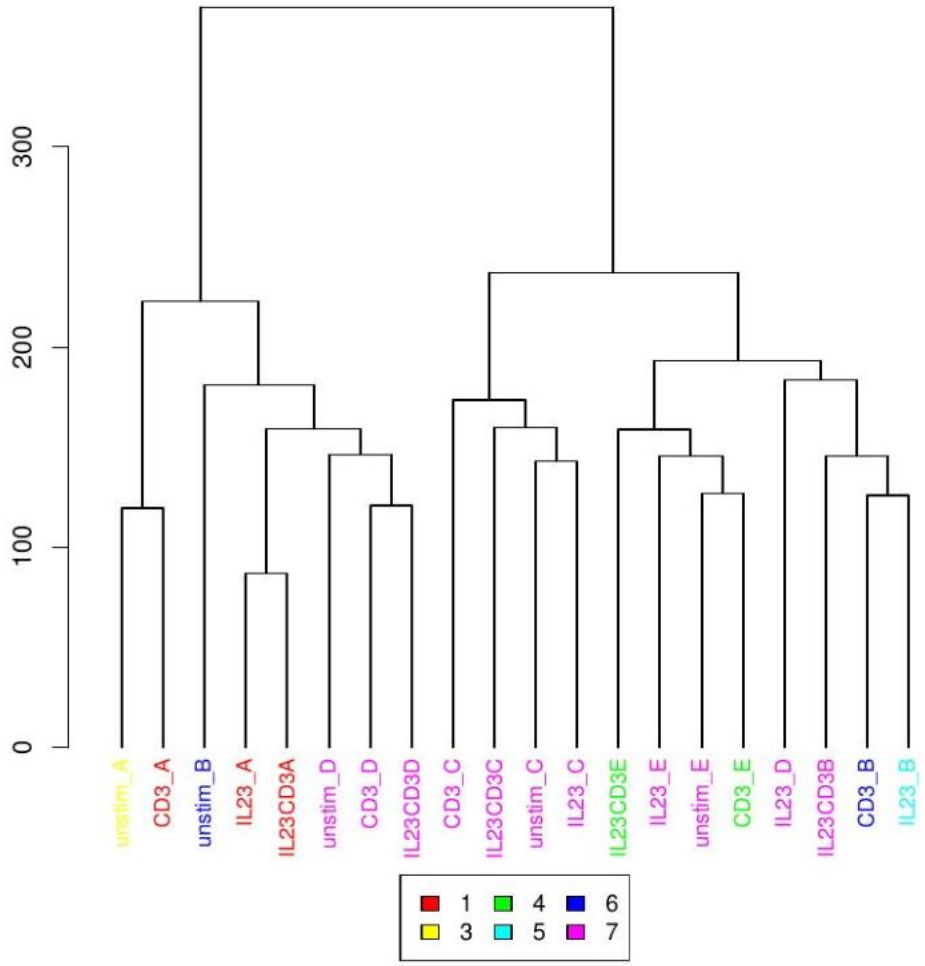


Figure 4.3.3. Hierarchical clustering of colonic explants samples (n=5) exposed to 4 conditions: unstimulated, anti-CD3 antibody, IL-23, and combination of IL-23 and anti-CD3 antibody. Each lane number is a represented with a distinct colour. (Figure created by Umar Niazi)

5.3. Differentially expressed genes

Having considered covariates differential gene expression between unstimulated and IL-23 exposed samples was calculated using the R based platform DSeq2 so producing a 'long list' of 11494 transcripts with associated fold changes, p-values, and false discovery rates.

5.3.1. Volcano plot

There are challenges involved in displaying enormous amounts of data to make it meaningful and displaying an excel list is not a useful or aesthetic approach. Displaying the data in volcano plots as seen in figure 5.3.1 below can be helpful. This image provides visual representation of the fold change (x-axis) and significance (y-axis) helping to identify transcripts which significantly up or down regulated. Analysing the figure below of DEGs from colonic explants exposed to standard media (unstimulated) v IL-23 added media there are a number of genes which are statistically significantly up and down regulated though few which also achieve fold changes over 1.5.

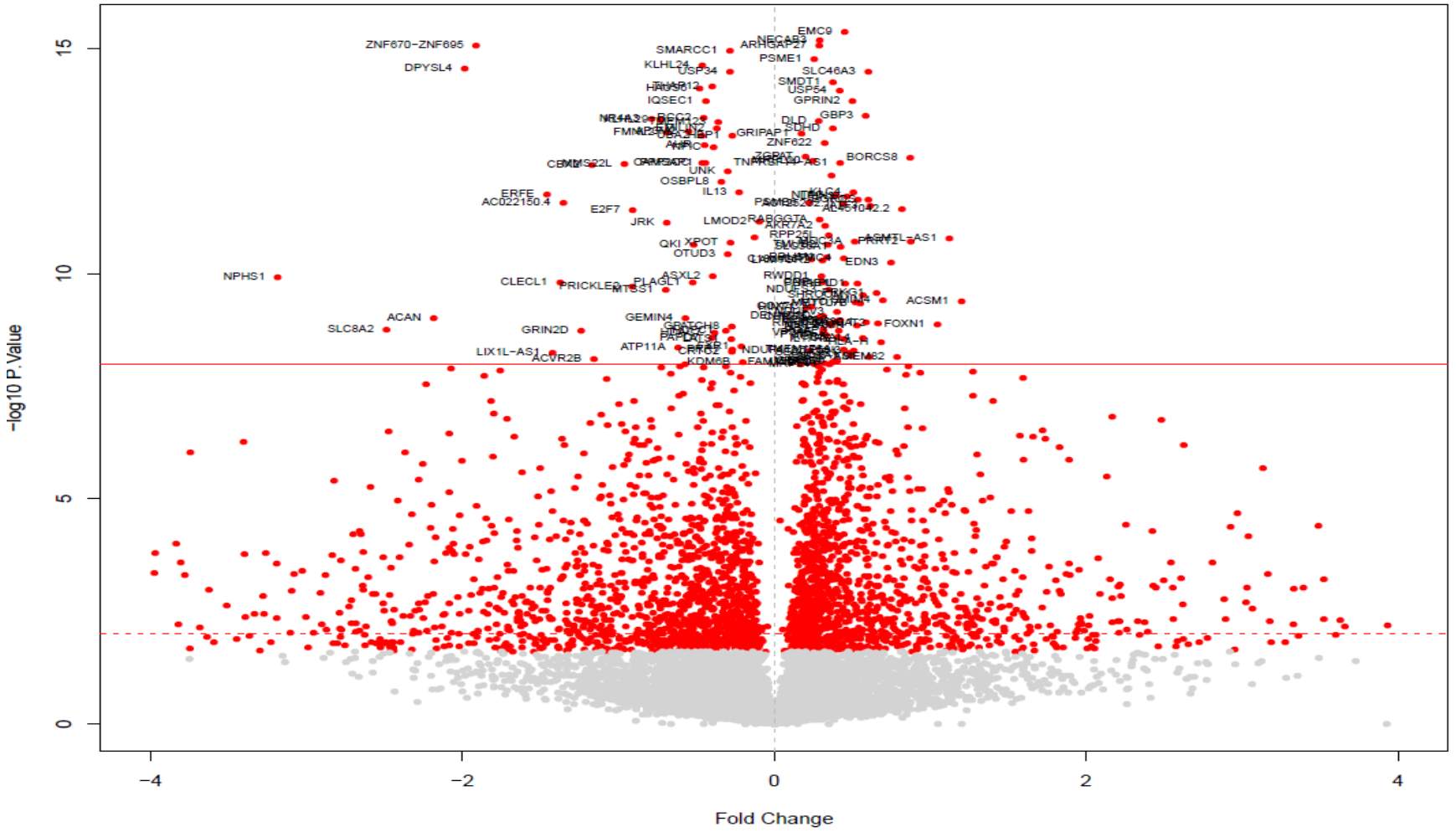


Figure 5.3.1. Volcano plot of DEGs from colonic explants from active UC IL-23 v unstimulated. X-axis = fold change in natural log and y-axis is adjusted p-value $-\log^{10}$. Red dots are genes with p <0.1, red dashed line unadjusted p <0.01 and solid red line top 5% of genes with padj <0.01.

5.3.2 Filtering transcripts of IL23 v unstimulated differentially expressed genes using padj<0.01 reveals 1226 differentially expressed genes

To elucidate more meaningful interpretation of the results we looked to sort the genes using filters.

Using a filter of padj <0.01 identified 1269 DEGs of which 43 were unmapped and removed from further analysis leaving 1226 – 618 upregulated and 608 downregulated.

Of interest amongst the most upregulated transcripts include relevant cytokine *IL-19* (padj = $1x10^{-5}$), cytokine receptors *IL23R* (padj = $2x10^{-3}$) & *IL10RB* (padj=$2x10^{-4}$)

Of the downregulated transcripts was integrin receptor *ITGB3* (padj=$6x10^{-4}$), cytokines *IL6* (padj=$6x10^{-3}$) & *IL13* (padj=$1x10^{-10}$), toll like receptot *TLR1* (padj=$5x10^{-6}$), cytokine receptor *IL10RA* (padj=$2x10^{-4}$).

5.3.3 Heatmap demonstrates variable IL-23 induced differentially expressed genes across samples

A further visual representation of differentially expressed genes is by using a heatmap. This allows visual comparison of samples across a range of differentially expressed genes as seen in figure 5.3.3. Strikingly, there is a large amount of variability amongst the samples even amongst those who have had the same culture conditions. Also interesting is that the closest sample by hierarchical clustering is from the same donor despite the influence of IL-23.

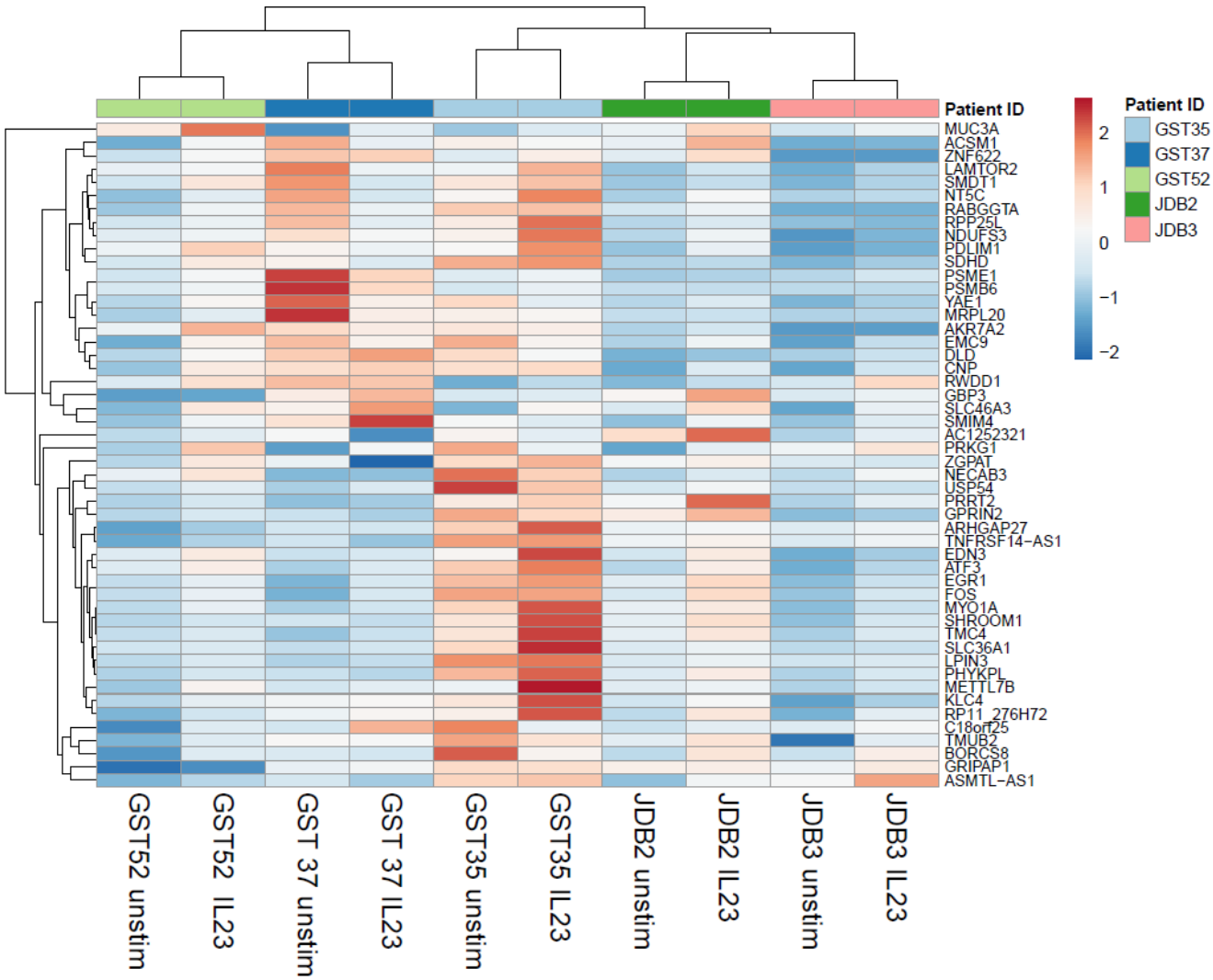


Figure 5.3.3. shows heatmap from top 50 most significantly upregulated differentially expressed genes from comparison of colonic explants from patients with UC cultured with standard media (unstimulated) v media with addition of IL-23. Hierarchical clustering has been applied to both the x-axis and y-axis.

5.3.4 Pathway analysis

Canonical pathway analysis of the DEGs discovered with filter padj <0.01 reveals 36 significantly ($p<0.05$) regulated pathways of which 17 are upregulated and 19 downregulated.

5.3.4.1 Upregulated pathways

Upregulated pathways are summarised in Figure 5.3.4.1. We had expected to see upregulated pathways associated with IL-23 activation such as Th17 pathway as well as T-cell proliferation pathways, pathogenic cytokine activation and cell to cell signalling. However, these pathways were not evident though there were several interesting pathway which warrant further evaluation.

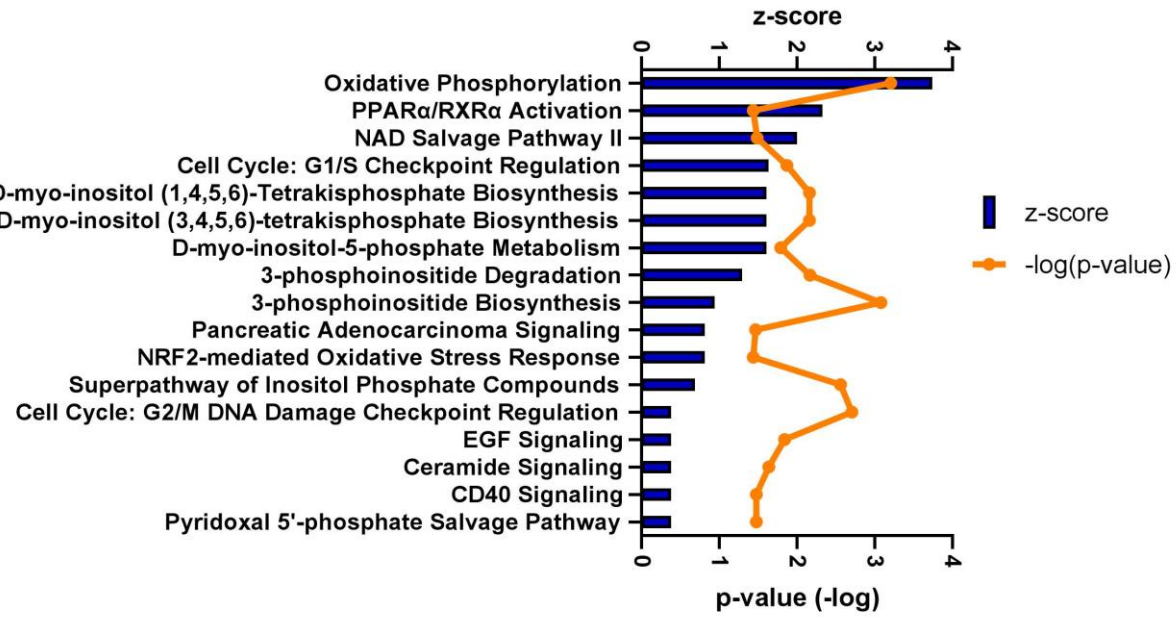


Figure 5.3.4.1. Statistically significant upregulated pathways derived from differentially expressed genes with padj<0.01 from colonic explants from patients with active UC co-cultured with standard media v addition of IL-23. Orange dots show p-value and blue bars are z-score (degree of activation of pathway)

5.3.4.2 Downregulated pathways

Downregulated pathways are shown in figure 5.3.4.2. Of the downregulated pathways there were 21 pathways which were significantly downregulated however only one of these had a significant z-score (<-2) which was the Aryl Hydrocarbon receptor. Other statistically significant pathways included relevant pathways of Th1 pathway, p53 signalling, T cell exhaustion and JAK/STAT pathway.

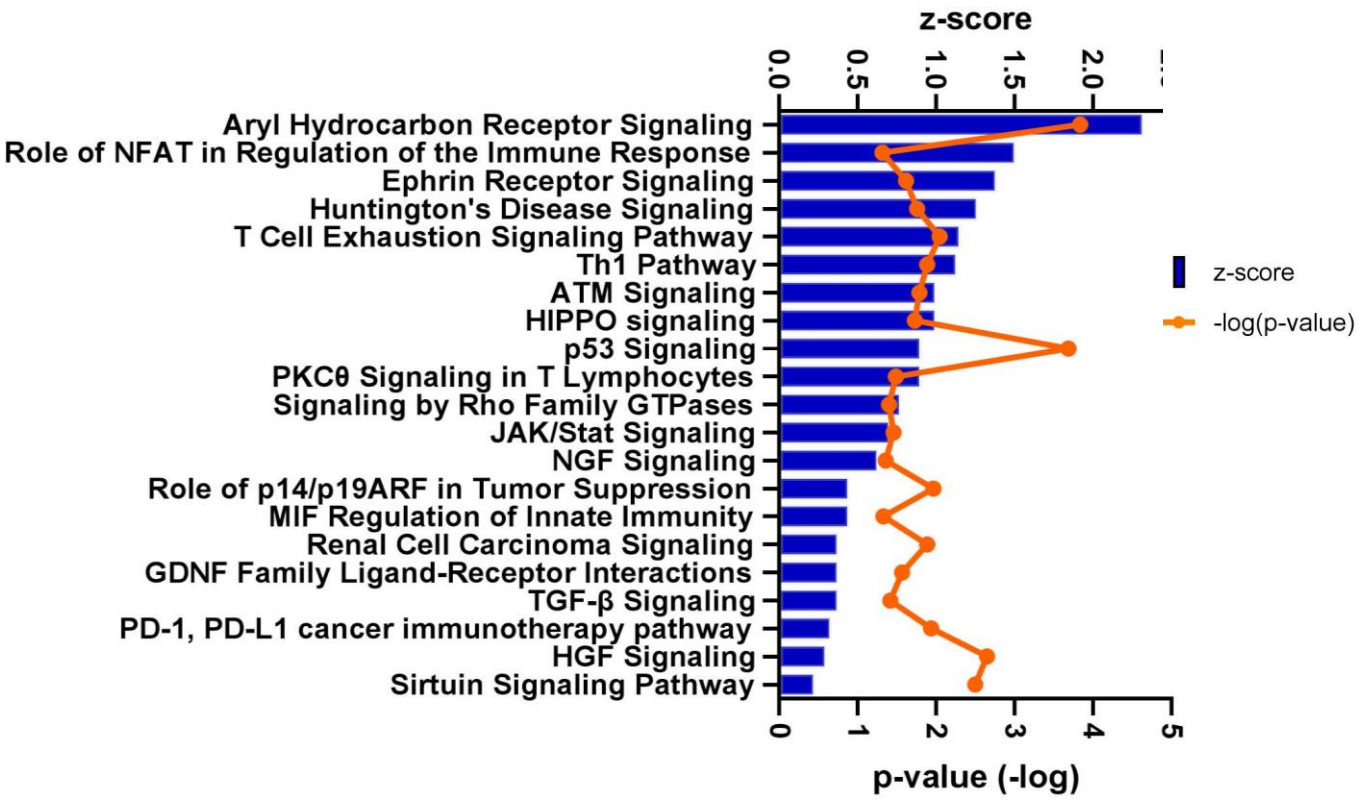


Figure 5.3.4.2. Statistically significant downregulated pathways derived from differentially expressed genes from colonic explants from patients with active UC co-cultured with standard media v addition of IL-23. Orange dots show p-value and blue bars are z-score (degree of activation of pathway)

5.3.4.3 Pathway analysis summary

Overall pathway analysis has highlighted interesting and relevant pathways but with contradictory effects of pro and anti-inflammatory effects.

PPARα was upregulated in our data. PPARα has been shown to be protective of intestinal inflammation in a DSS induced mouse model of colitis where Wy-14643 (a PPARα agonist) attenuated colitis and produced less inflammatory cytokines IFNγ, TNFα, IL-1β, IL-6 (Azuma et al. 2010). A further DSS mouse model also showed attenuation of colitis with rSj16 (a protein produced by E. Coli) by inhibiting PPARα pathway signalling (Wang et al. 2017).

PPARγ has been more widely explored in humans especially in UC and acts similarly to its α isoform and forms a complex with RXRα. Studies have shown a marked decreased expression in the colons of patients with UC (Dubuquoy et al. 2003) and have been shown to reduce pro-inflammatory cytokines by attenuating NFκB in mouse models of colitis (Desreumaux et al. 2001). Furthermore PPARγ inhibitor rosiglitazone has been trialled in UC and showed improved clinical response and clinical remission though no improvement endoscopically (Lewis et al. 2008). However, further exploration as a potential therapy has been prohibited by the poor safety profile with an increased risk of

myocardial infarction. A novel 5-ASA analogue with potent PPARγ agonist properties named GED-0507-34 Levo was found to abrogate inflamed driven intestinal fibrosis in mouse models (Speca et al. 2016) and a phase 2 clinical trial was organised though failed to recruit and was abandoned (Medicine 2017).

The NAD salvage pathway was upregulated in our data and has been implicated in IBD as intracellular nicotinamide phosphoribosyltransferase (NAMPT), a rate limiting enzyme of the NAD salvage pathway, has been found to be overexpressed in the blood and colonic tissue of patients with IBD (Moschen et al. 2007). Furthermore FK866, a small molecule inhibitor of NAMPT has been shown to ameliorate colitis and reduced mucosal in a DSS mouse model and when co-cultured with LPMCs of patients with IBD showed significant decrease in TNF, IL-6 and IFNγ (Gerner et al. 2018).

We found the Aryl hydrocarbon pathway to be downregulated. This is in contrast to the literature which shows that IL-23 would upregulate AhR in murine neutrophils (Chen et al. 2016).

Furthermore, expected pathway upregulation such as the Th17 pathway, T cell activation, cell to cell interactions and cytokine upregulation were not seen.

5.3.5 Upstream regulators

Analysis of upstream regulators can be useful to identify factors which have contributed to the transcriptomic changes. This is a useful exercise as it can identify mediators which share similar biological activity to IL-23.

5.3.5.1 Positive upstream regulators

Overall, 15 factors were identified with $p<0.05$ and z-score >2 including 4 drugs (nitric oxide, nilotinib, imipramine and arsenic) as well as prolactin and interferon α receptor.

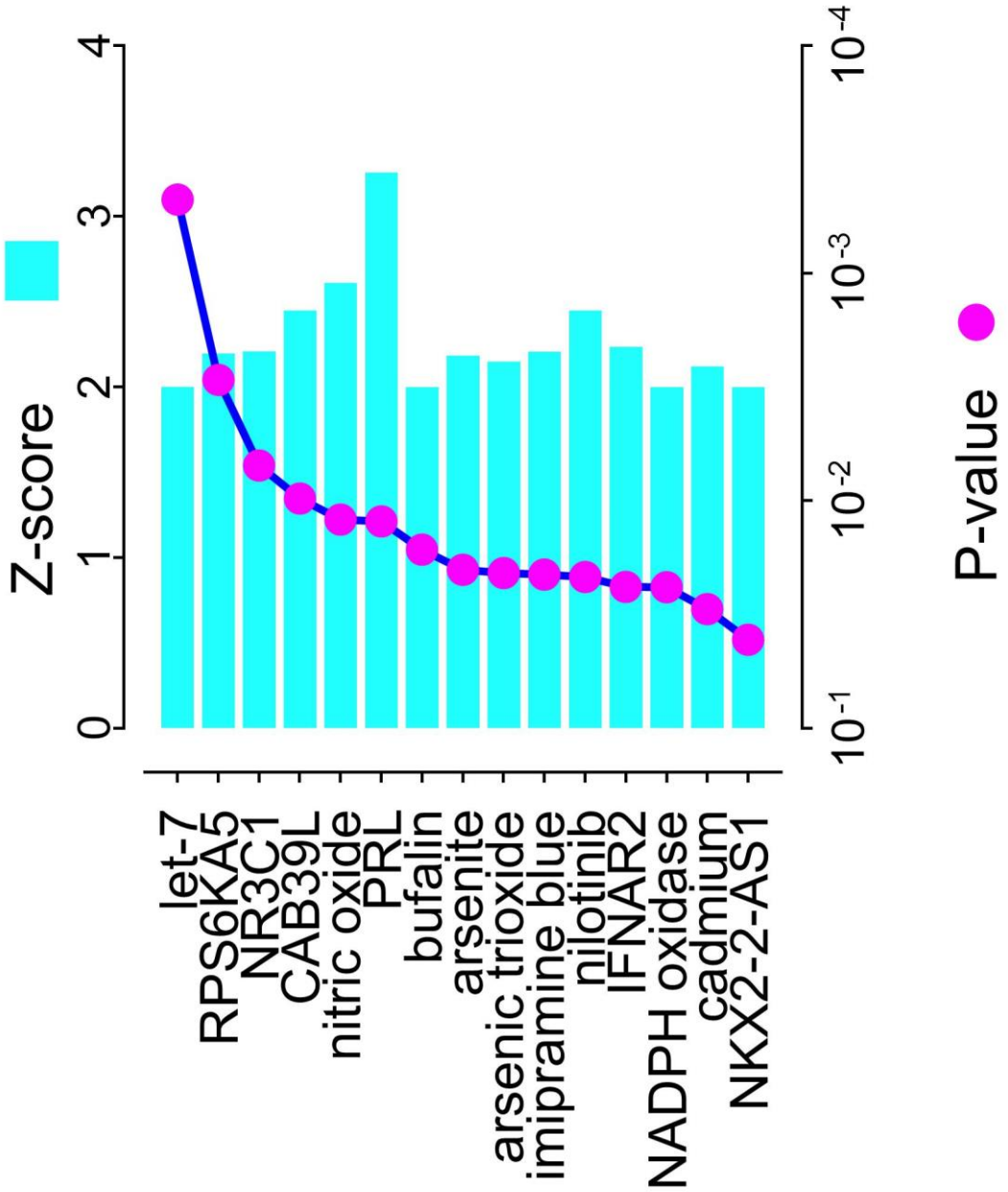


Figure 5.3.5.1. Graph showing positive upstream regulators of IL-23 stimulated versus unstimulated colonic explants from patients with ulcerative colitis. Differentially expressed genes filtered using fdr<0.01 and analysed using Ingenuity Pathway Analysis. Displayed with z-scores >2 (blue bars) and p<0.05 (purple dot and lines).

Arsenic has been used since 1960s for treatment of refractory proctitis with some evidence of benefit (Kiely et al. 2018).

Nilotinib, a tyrosine kinase inhibitor, has been shown to ameliorate colitis in a mouse model of colitis (Ataca et al. 2013) though no trials have performed in humans.

IFNAR2 encodes for interferon α receptor 2 which binds type 1 interferons which have been associated with anti-inflammatory effects and amelioration of colitis in mouse models. Furthermore, it has been shown to inhibit the Th17 differentiation and downregulates IL-23 (Shinohara et al. 2008; Ramgolam et al. 2009).

5.3.5.2 Negative upstream regulators

Using the same list of upstream regulators, the list can be inverted to identify factors which would inhibit the effect of the transcriptomic change. Therefore, these factors may be useful in identifying novel drug targets or identifying areas which require further exploration.

8 factors were identified with $p<0.05$ and z-score <-2 including retinoid ST1926, transcription factor TBX2 and kinase MAPK1 as shown in figure 5.4.5.2.

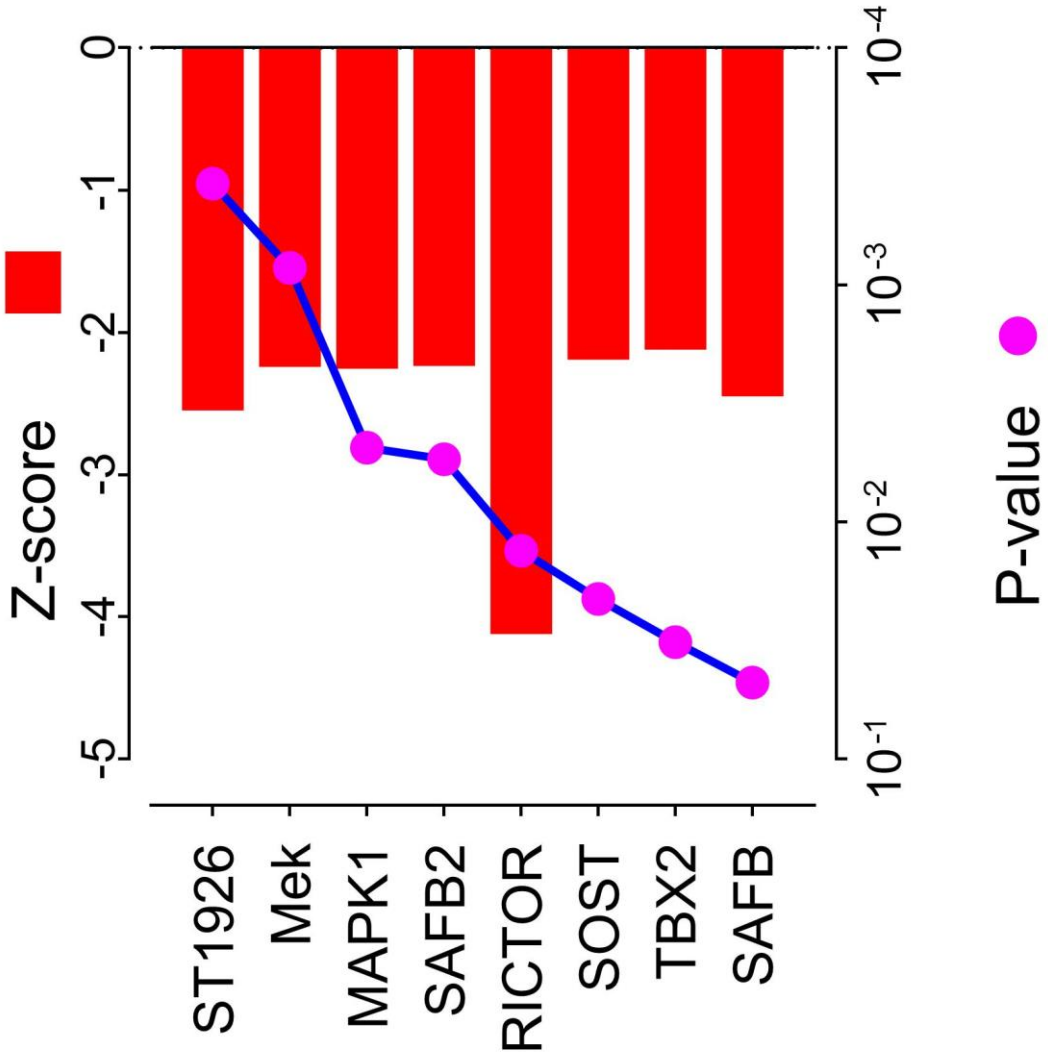


Figure 5.3.5.2. Graph showing negative upstream regulators of IL-23 stimulated versus unstimulated colonic explants from patients with ulcerative colitis. Differentially expressed genes filtered using padj<0.01 and analysed using Ingenuity Pathway Analysis. Displayed with z-scores >2 (blue bars) and p<0.05 (purple dot and lines).

Mitogen activated protein kinases (MAPK) are activated by diverse extracellular and intracellular stimuli, and thereby they play an essential role in connecting cell-surface receptors to changes in transcriptional programs. The MAPK signalling pathways regulate a wide range of cellular activities including upregulating IBD relevant transcription factors NFκB and STAT molecules and have been implicated in the pathogenesis of several diseases, including IBD (Coskun et al. 2011; Broom et al. 2009). Several drugs have been created against p38 MAPK and have shown ex vivo to reduce inflammatory cytokines (Gruenbaum et al. 2009). Randomised control trials have shown clinical but not endoscopic benefit in UC over placebo (Travis et al. 2005) but 2 studies in CD failed to find benefit above placebo (Dotan et al. 2010; Schreiber et al. 2006).

However, given its mode of action MAPK1, we might have expected it to be a positive regulator in our experiment.

ST1926 is atypical retinoid which has been shown to have anti-tumour properties in several cancers including haematological and colorectal. They act by inhibiting cell proliferation and inducing apoptosis (Abdel-Samad et al. 2018; Nasr et al. 2015). However, they have not been investigated in IBD and so their utility in treating IBD is unclear and corroborating studies would be required before further conclusions can be drawn.

5.4 Conclusion

The analysis of colonic explants from patients has raised more questions than answers them.

RT-qPCR did not show induction of IL-17A which had been expected. However, IL-17A induction was not seen when LPMCs were stimulated with IL-23 so whilst surprising given that it is a canonical downstream cytokine, this result was not unexpected. Lack of IL-17A induction was replicated with RNAseq.

We found that IL-22 was significantly upregulated when investigated with RT-qPCR but relevant pro-inflammatory pathways such as the Th17 pathway or T cell activation were not found. Instead, we found activation of IBD relevant oxidative phosphorylation and PPARγ pathways and downregulation of aryl hydrocarbon receptor pathways. Together these findings do not show a coordinated pro-inflammatory pattern of effect of IL-23 as expected but a series of opposing unexpected pro and anti-inflammatory pathways.

This transcript and pathway analysis of the explant experiments did not show the similarities to the LPMC experiments that we had hoped. Potential reasons for these findings are discussed in detail further in 7.1.8

The lack of correlation of the explant analysis to the LPMC analysis as well as the lack of a coordinated pro-inflammatory response led to the decision that we would not create a gene signature using the explant experiment data which are extensively used in chapter 6 using solely LPMC data.

6. Utilising transcriptional derived signatures to predict response to therapies in IBD

In chapter 4 we demonstrated that when IL-23 is exposed to lamina propria mononuclear cells from colonic biopsies from patients with active ulcerative colitis it produces a profound Th17 predominant transcriptomic effect. Therefore, we explored whether we could take these transcriptional changes and create it into a clinically meaningful biomarker. Given the lack of biomarkers available in clinical practice it would be highly useful to clinicians to be able to better inform patients of the chances of success of each medical option.

We hypothesised that transcripts that were regulated by IL-23 would be enriched in lamina propria mononuclear cells and whole biopsy specimens in patients with active UC versus healthy controls and inactive UC.

Furthermore, we hypothesized that patients with the greatest magnitude of enrichment of IL-23 responsive transcripts would respond most favourably to anti-IL12p40 therapy and respond least favourably to anti-TNF.

In order to evaluate these hypotheses, we developed various gene signatures from the DEGs from colonic explants and LPMCs which were experimented with including up and down regulated, numerical cut offs (e.g. top 100 statistically significant) as well as statistical cut offs (e.g. fdr < 0.01, p < 0.01).

We utilised Gene Set Variation Analysis (GSVA) to assess the enrichment of our gene sets across a set of samples. This method takes a gene signature and interrogates the abundance of expression of these genes within whole genome RNA sequencing (microarray or RNAseq) which has been obtained either from reposited datasets, datasets generated from our own institution or from industry collaborators. The samples are compared to other samples within the group and an enrichment score is calculated from -1 to +1 to indicate the degree of enrichment of that sample within the group. The enrichment scores are then combined with outcome data to permit comparison of the enrichment sores in the groupings.

GSVA works optimally with a gene signature of around 100 genes. We ran a number of preliminary enrichment scores using a host of different gene signatures and settled on $p<0.01$ which satisfies the need for around 100 genes to optimally work GSVA and

Reposited datasets are publicly available datasets and are described in detail in 3.14.

6.1. Comparison of enrichment scores in active UC v healthy control

To evaluate the validity of the gene signatures we first evaluated to see if they could differentiate active UC from healthy controls

6.1.1. IL23 LPMC derived gene signature

The IL23 LPMC signature consisted of 112 upregulated transcripts with $p<0.01$

6.1.1.1. No statistical difference between healthy control and active UC in KCL cohort using IL23 LPMC gene signature

Our KCL cohort consisted of 16 patients with active UC (endoscopic Mayo 2 or 3) none of which were on biologics as well as 6 healthy controls. RNAseq was performed on a single colonic biopsy and data used as a dataset.

In the KCL cohort there was no significant difference between enrichment scores of healthy control and active UC ($p=0.11$) as seen below in figure 6.1.1.1.

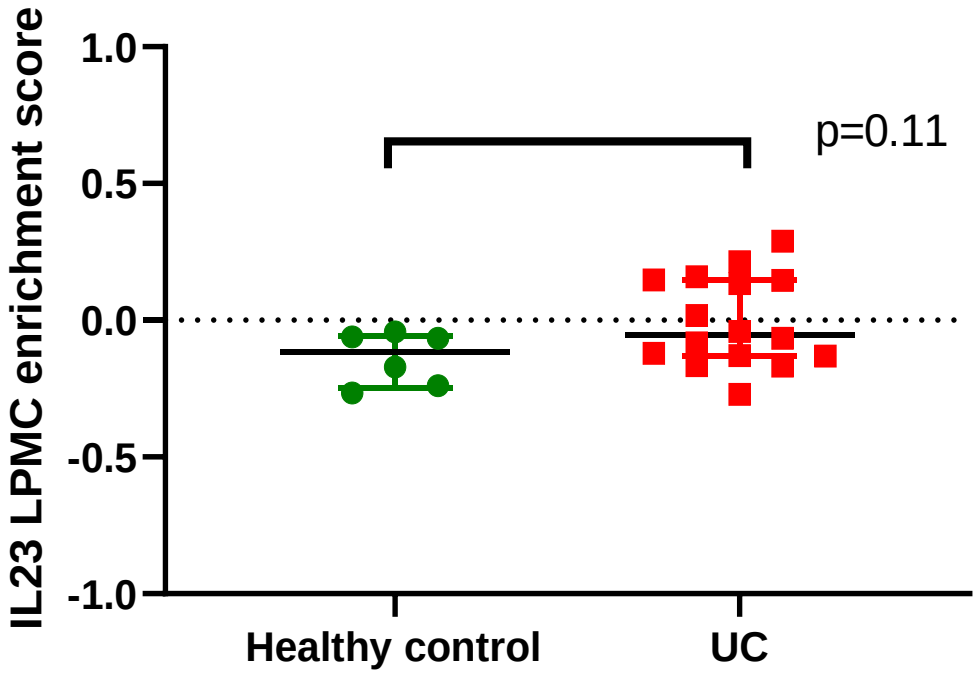


Figure 6.1.1.1. graph enrichment scores from KCL dataset with IL23 LPMC gene signature generated from upregulated differentially expressed genes from IL-23 stimulated LPMCs from patients with UC with filter of p<0.01. HC (n=6, median = -0.012, IQR: -0.25 – -0.06) and UC (n=16, median= -0.05, IQR: -0.13 – 0.15). No statistical difference between HC and UC (p=0.11, Mann-Whitney 2 tailed test). Data displayed with median and interquartile range bars.

6.1.1.2. Active UC is significantly more enriched than healthy control and inactive UC in GSE59071 using the IL23 LPMC gene signature

Using reposited dataset GSE59071 (Vanhove et al. 2015) calculated enrichment scores using the IL23 LPMC signature as shown below in figure 6.1.1.2. Active UC has a statistically significant ($p<0.00001$) increase in enrichment score compared to both healthy controls and inactive UC. There was no difference between healthy control and inactive UC.

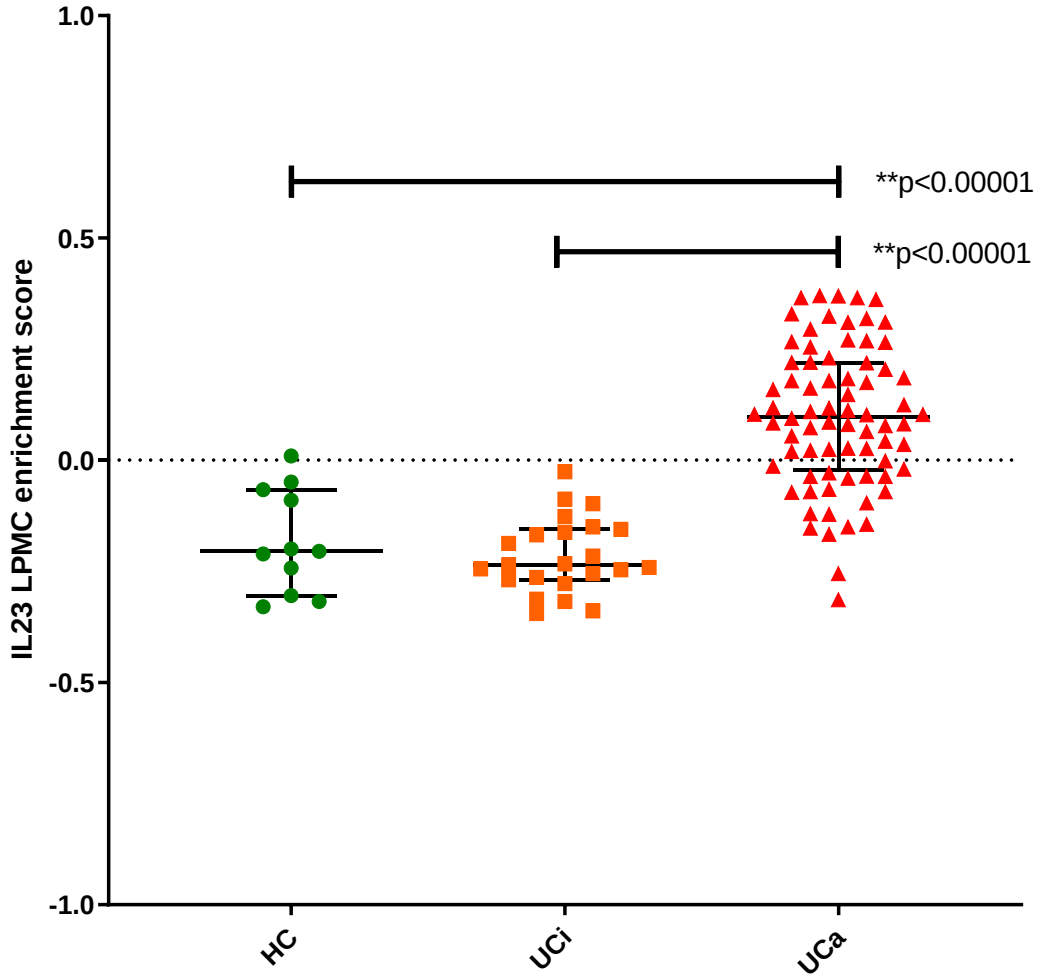


Figure 6.1.1.2. graph enrichment scores from reposited data set GSE59071 with IL23 LPMC gene signature generated from upregulated differentially expressed genes from IL-23 stimulated LPMCs from patients with UC with filter of p<0.01. Healthy control (HC) (n= 11, median= -0.21, IQR: -0.30-0.07), inactive UC (iUC) (n=23, median= -0.24, IQR: -0.273 – 0.16) and active UC (aUC) (n= 74, median= -0.10, IQR: -0.02 – 0.22). Statistically significant increase in GSVA score was seen between HC and aUC (p<0.00001 Dunn's multiple comparison test using 1 way ANOVA) and iUC and aUC (p<0.00001 Dunn's multiple comparison test using 1 way ANOVA). Data displayed with median and interquartile range bars.

6.1.1.3. Conclusions

Th IL23 LPMC gene signature is highly significantly enriched in active UC in the largest reposited database as hypothesised. Statistically significant differences were not seen in the KCL cohort though this is likely due to the small sample size.

6.1.2. IL23 colonic explant signature

To generate the IL23 colonic explant signature we took the 100 most upregulated transcripts with padj<0.01.

6.1.2.1. Active UC is statistically significantly less enriched than healthy controls in dataset GSE59071

Enrichment scores were calculated using reposited dataset GSE59071 (Vanhove et al. 2015) and is shown below in figure 6.1.2.1. This shows enrichment scores of healthy controls, inactive UC (iUC) and active UC (aUC). Unexpectedly, active UC had statistically lower enrichment score compared to healthy controls (p=0.02, Kruskal-Wallis multiple comparisons) but no significant difference was seen between inactive UC and healthy controls.

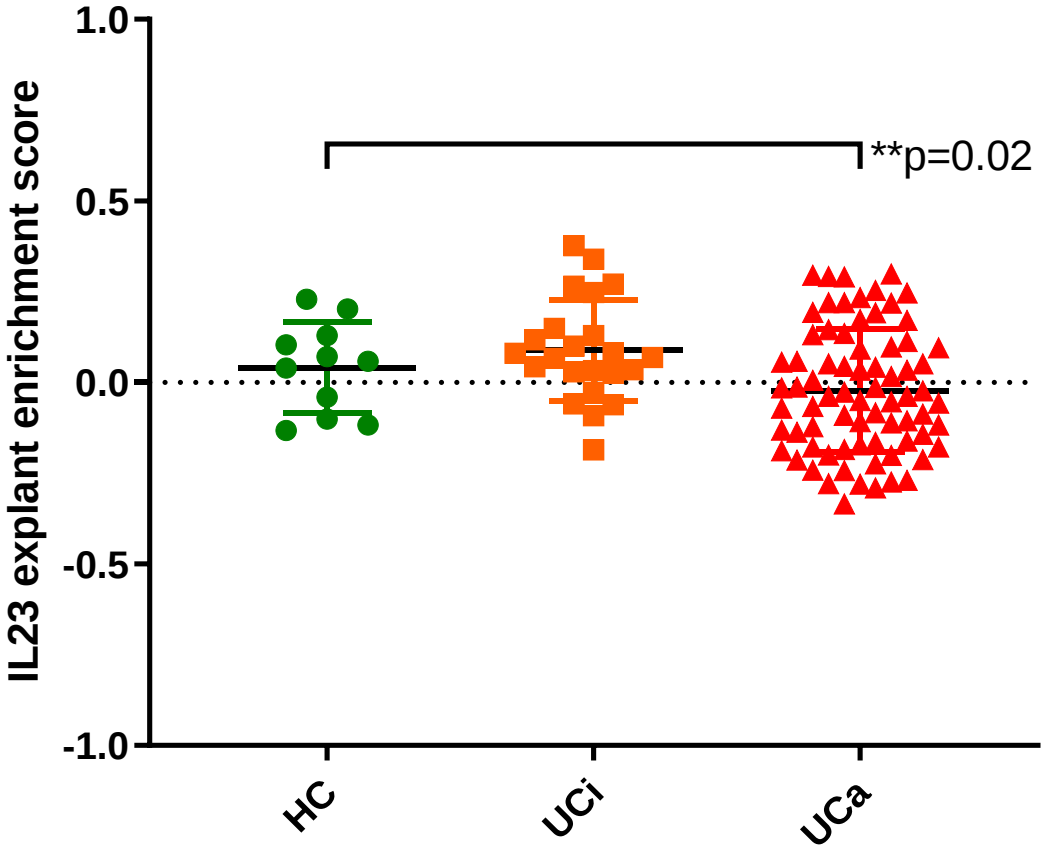


Figure 6.1.2.1. graph enrichment scores from reposited data set GSE59071 with gene list generated from top 100 differentially expressed genes from IL-23 stimulated explants from patients with UC with filter of fdr<0.01. Healthy control (HC) (n= 11, median= 0.06, IQR: -0.10-0.13), inactive UC (iUC) (n=23, median= 0.07, IQR: 0.03 – 0.14) and active UC (aUC) (n= 74, median= -0.03, IQR: -0.16 – 0.10). Statistically significant decrease in GSVA score was seen between HC and aUC (p=0.02 Dunn's multiple comparison test using 1 way ANOVA). Data displayed with median and interquartile range bars.

6.1.2.2. No statistical difference in enrichment scores comparing healthy control v active UC in KCL dataset using IL23 colonic explant signature

We compared enrichment scores using the IL23 colonic explant derived gene signature derived from our own cohort of patients that we have performed RNAseq upon. This did not show any statistical difference in enrichment scores as shown in figure 6.1.2.2. below.

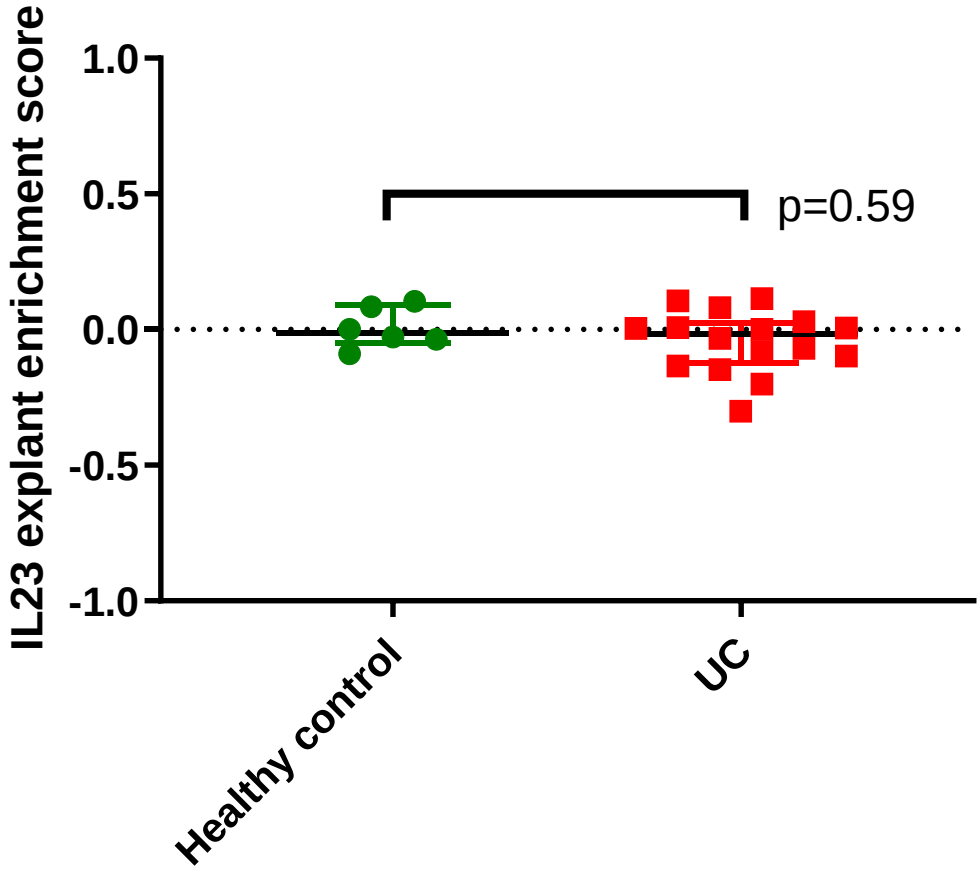


Figure 6.1.2.2. graph enrichment scores from KCL patient cohort with gene list generated from top 100 upregulated differentially expressed genes from IL-23 stimulated explants from patients with UC with filter of fdr<0.01. HC (n=6, median = -0.013, IQR: -0.050 – 0.090) and UC (n=16, median= -0.016, IQR: -0.125 – 0.023). No statistical difference between HC and UC (p=0.59, Mann-Whitney 2 tailed test). Data displayed with median and interquartile range bars.

6.1.2.3. Conclusions

The Vanhove dataset (GSE59071) dataset active UC had a lower enrichment score than healthy control which was unexpected. We had hypothesised that there would be a higher enrichment score in active disease compared to healthy control.

It is disappointing that the KCL dataset could not separate healthy control from active UC though numbers are small and therefore interpretations should be cautious.

6.2. Comparison of enrichment scores in anti-IL12p40

To evaluate our hypothesis that our IL23 LPMC gene signature would predict response to anti-IL12p40 molecule we collaborated with commercial colleagues in Jansenn who utilised our gene signature to calculate enrichment scores in their UNIFI dataset of 550 patients.

The UNIFI trial was a large international multi-site placebo controlled double blinded randomised control trial investigating either placebo or ustekinumab with 2 initial doses. 186 patients received placebo, 180 patients received ustekinumab 130mg and 184 patients received 6mg/kg dose. All patients were reviewed at week 8 endoscopically and clinically and enrichment scores were also calculated for patients at week 8 as well as week 0. They also provided biochemical and clinical data.

6.2.1. Enrichment scores positively correlate with total Mayo scores

Initial analysis was to see if there was any correlation of enrichment scores with biochemical and demographic data using all the patients (i.e., placebo and ustekinumab). As shown in figure 6.2.1. there is weak positive correlation which was statistically significant with CRP (R^2=0.008, p=0.04), faecal calprotectin

(R^2=0.008, p=0.04) and faecal lactoferrin (R^2=0.010, p=0.03). Furthermore, there was weak positive correlation which was highly statistically significant when enrichment scores were compared to total Mayo scores (R^2=0.060, p<0.0001).

There is no correlation between enrichment scores and duration of disease.

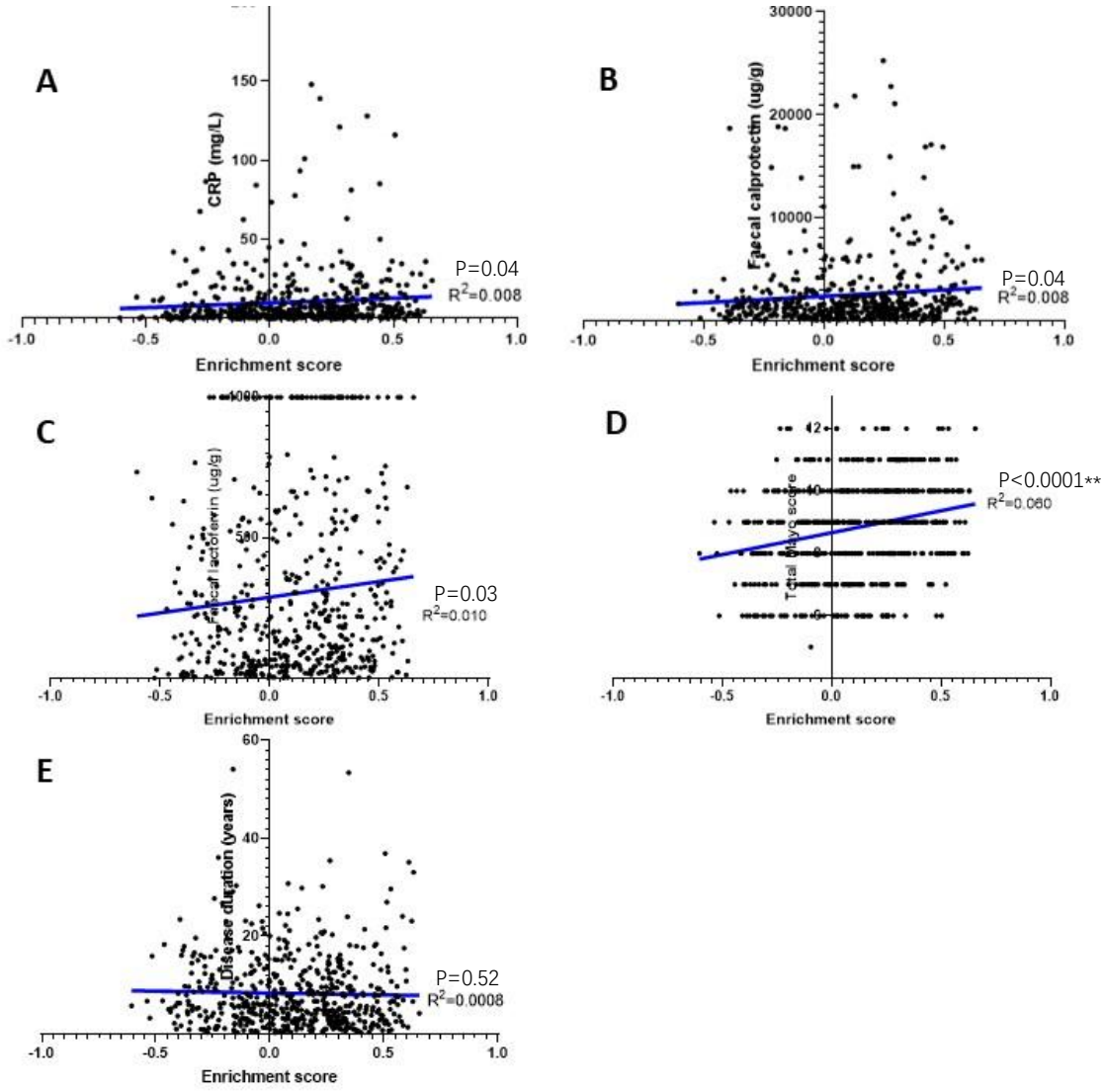


Figure 6.2.1. graphs of IL23 LPMC enrichment scores of patients entering UNIFI trial at week 0 v biochemical and demographic data. Straight line non-linear regression calculated using Least squares regression. A – CRP (mg/L), n=544, R^2=0.008, p=0.04; B – faecal calprotectin (µg/g), n=512, R^2=0.008, p=0.04; C – faecal lactoferrin (µg/g), n=516; R^2=0.010, p=0.03; D – total Mayo score, n=550, R^2=0.060, p<0.0001; E – disease duration (years), n=550, R^2=0.0008, p=0.52.

6.2.2 IL23-responsive transcripts (LPMC signature) are significantly enriched in pre-treatment (baseline) colonic biopsies from UC patients who did not achieve endoscopic remission and mucosal healing at week 8 following induction with ustekinumab

We analysed enrichment scores from patients using endoscopic healing (Mayo endoscopic sub score of 0 or 1) and mucosal healing (histologic improvement (defined as neutrophil infiltration in <5% of crypts, no crypt destruction, and no erosions, ulcerations, or granulation tissue) data.

As shown in figure 6.2.2. there is a significant decrease in IL23 LPMC enrichment scores of patients who achieve endoscopic healing (p=0.004) and mucosal healing (p=0.01). ROC analysis calculation shows an AUC of 0.60 for both endoscopic healing and mucosal healing.

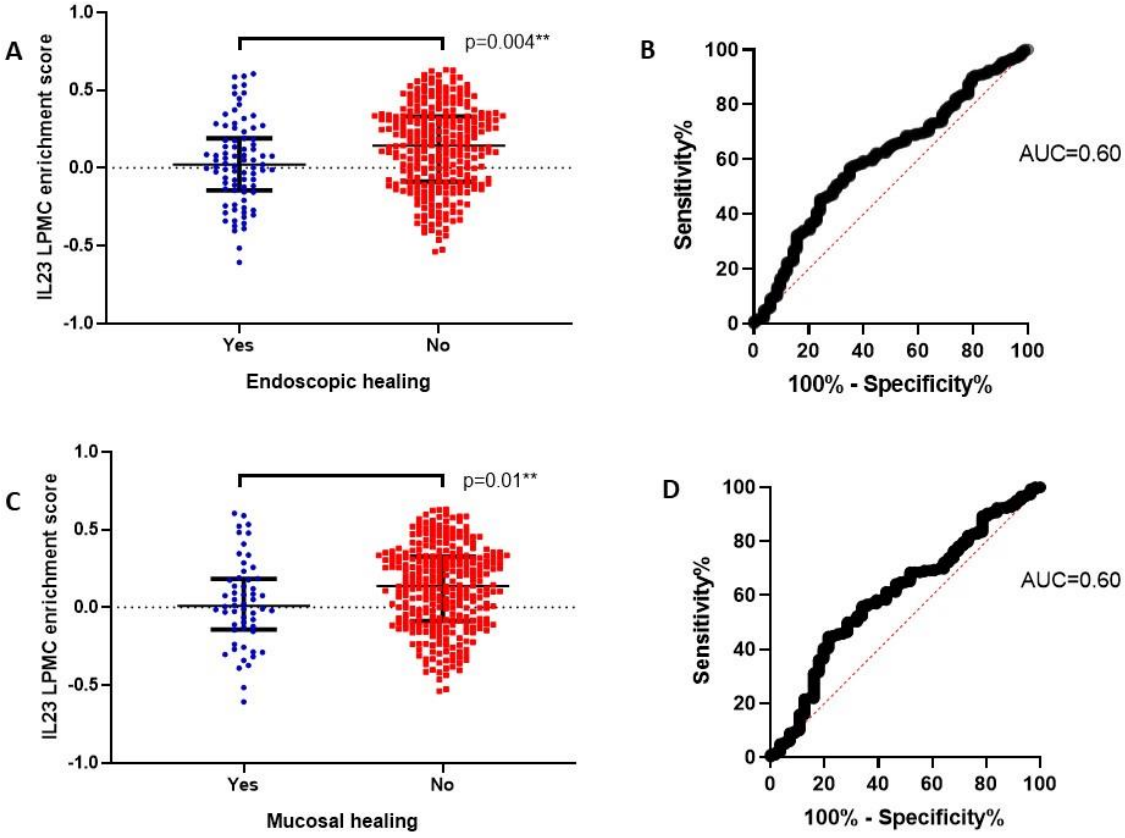


Figure 6.2.2. graphs and area under the curves of IL23 LPMC enrichment scores in patients who had not achieved endoscopic healing or mucosal healing who had received ustekinumab in UNIFI trial at week 8

A: graph of IL23 LPMC enrichment scores in patients who had an had not achieved endoscopic healing who had received ustekinumab in UNIFI trial at week 8 (yes: n= 83, median=0.02, IQR=-0.14 – 0.19, no n= 281, median=0.14, IQR=-0.08 – 0.33, p=0.004 2-tailed Mann-Whitney test).

B: ROC analysis of IL23 LPMC enrichment scores in patients with endoscopic healing at week 8 who had received ustekinumab in UNIFI trial, AUC=0.60.

C: graph of IL23 LPMC enrichment scores in patients who had and had not had mucosal healing who had received ustekinumab in UNIFI trial at week 8 (yes: n= 56, median=0.00, IQR=-0.14 – 0.18, no: n= 302, median=0.14, IQR=-0.09 – 0.33, p=0.01 2-tailed Mann-Whitney test).

D: ROC analysis of IL23 LPMC enrichment score in patients in clinical remission at week 8 who had received ustekinumab in UNIFI trial, AUC=0.60.

6.2.3. IL23-responsive transcripts (LPMC signature) are significantly enriched in pre-treatment (baseline) colonic biopsies from UC patients who did not have deep remission as well as clinical remission plus endoscopic healing at week 8 following induction with ustekinumab

We performed analysis using data from patients with deep remission (clinical remission and mucosal healing) as well as patients who achieved clinical remission and endoscopic healing as is summarised in figure 6.2.3.

Graphs A and C show a statistical significant decrease in IL23 LPMC enrichment scores in patients who have achieved deep remission (p=0.01) and patients who achieved clinical remission and endoscopic healing (p=0.01). Furthermore ROC analyses (B & D) show AUC of 0.62.

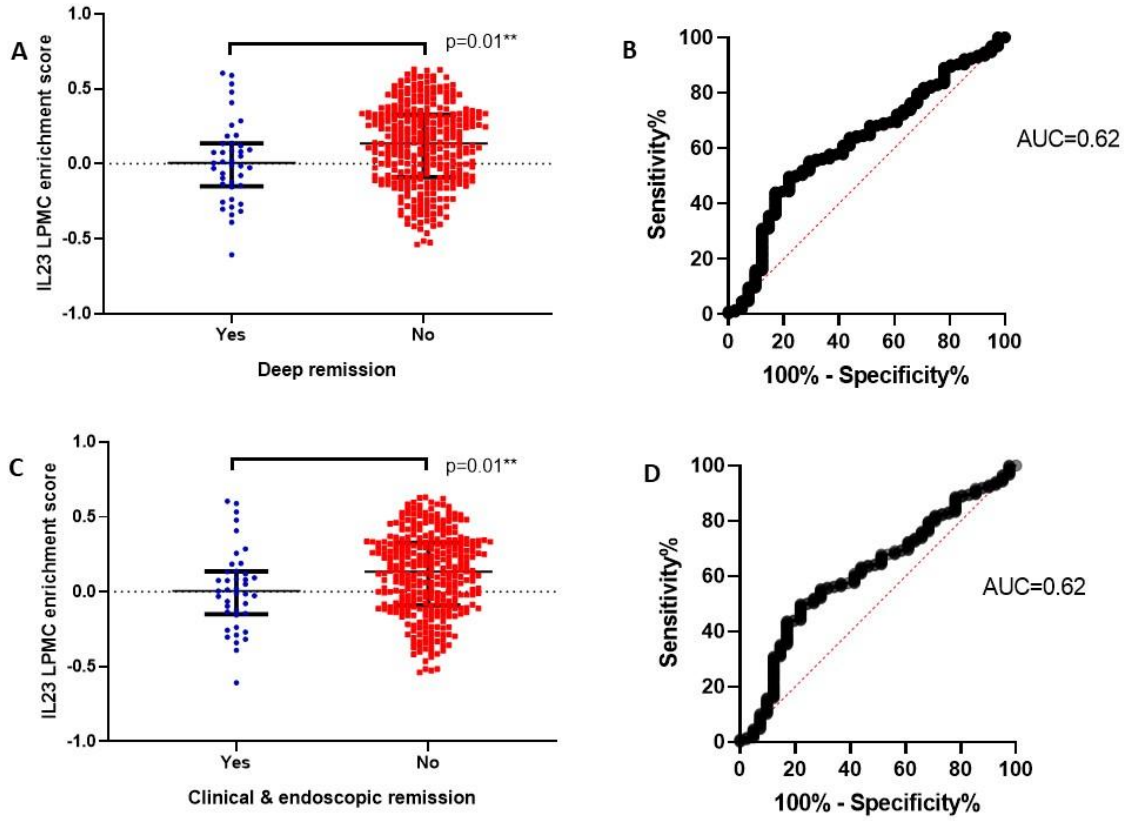


Figure 6.2.3. graphs and area under the curves of IL23 LPMC enrichment scores in patients who had not achieved deep remission or clinical remission and endoscopic healing who had received ustekinumab in UNIFI trial at week 8

A: graph of IL23 LPMC enrichment scores in patients who had an had not achieved deep remission who had received ustekinumab in UNIFI trial at week 8 (yes: n= 41, median=0.01, IQR=-0.15 – 0.14; no n= 320, median=0.13, IQR=-0.09 – 0.33; p=0.01 2-tailed Mann-Whitney test).

B: ROC analysis of IL23 LPMC enrichment scores in patients with endoscopic healing at week 8 who had received ustekinumab in UNIFI trial, AUC=0.62.

C: graph of IL23 LPMC enrichment scores in patients who had and had not had clinical and endoscopic remission who had received ustekinumab in UNIFI trial at week 8 (yes: n= 41, median=0.01, IQR=-0.15 – 0.14; no: n= 327, median=0.14, IQR=-0.09 – 0.33, p=0.01 2-tailed Mann-Whitney test).

D: ROC analysis of IL23 LPMC enrichment score in patients in clinical remission at week 8 who had received ustekinumab in UNIFI trial, AUC=0.62.

6.2.4. IL23-responsive transcripts (LPMC signature) are significantly enriched in pre-treatment (baseline) colonic biopsies from UC patients who do not achieve clinical remission at week 8 following induction with ustekinumab

We analysed the relationship between clinical remission (total Mayo score of ⩽2 and no sub score >1) at week 8 in patients who had received ustekinumab (i.e., placebo was excluded) which is detailed below in figure 6.2.4.

However, there was a strong statistical difference between patients who were in clinical remission at week 8 (p=0.005). Interestingly, patients who were in clinical remission had significantly lower enrichment scores than patients not in clinical remission which was contrary to our hypothesis. ROC analysis showed an AUC of 0.62.

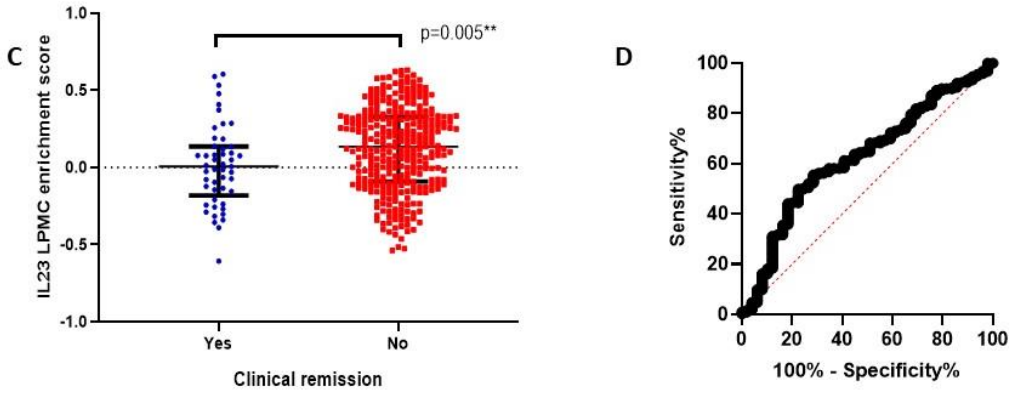


Figure 6.2.4. graphs and area under the curves of IL23 LPMC enrichment scores in patients who had not achieved clinical remission who had received ustekinumab in UNIFI trial at week 8

A: graph of IL23 LPMC enrichment scores in patients who had and had not had clinical remission who had received ustekinumab in UNIFI trial at week 8 (yes: n= 49, median=0.00, IQR=-0.18 – 0.14, no: n= 315, median=0.14, IQR=-0.09 – 0.33, p=0.005 2-tailed Mann-Whitney test).

B: ROC analysis of IL23 LPMC enrichment score in patients in clinical remission at week 8 who had received ustekinumab in UNIFI trial, AUC=0.62.

6.2.5. IL23-responsive transcripts (LPMC signature) are not significantly enriched in pre-treatment (baseline) colonic biopsies from UC patients who had a clinical response at week 8 following induction with ustekinumab

We analysed the relationship between patients who had had a clinical response (a decrease in the total Mayo score of at least 30% and of at least 3 points from baseline, with an accompanying decrease of at least 1 point on the Mayo rectal bleeding sub score or a rectal bleeding sub score of 0 or 1) at week 8 in patients who had received ustekinumab (i.e. placebo was excluded) which is detailed below in figure 6.2.5.

There was no statistical difference in IL23 LPMC enrichment scores in patients who had clinical response or not. The corresponding ROC analysis had an AUC of 0.50.

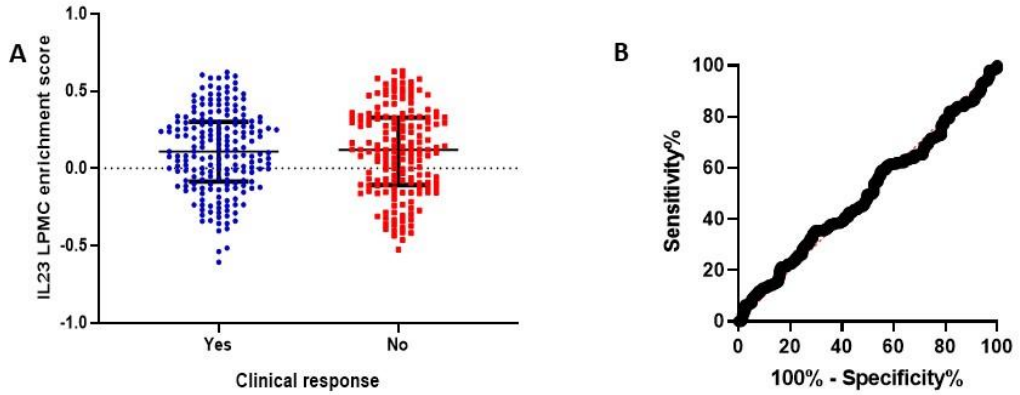


Figure 6.2.5. graph and area under the curves of IL23 LPMC enrichment scores in patients who had and had a clinical response at week 8 who had received ustekinumab in UNIFI trial

A: graph of IL23 LPMC enrichment scores in patients who had an had not had clinical response who had received ustekinumab in UNIFI trial at week 8 (yes: n= 195, median=0.11, IQR=-0.09 – 0.30, no: n= 169, median=0.12, IQR=-0.11 – 0.33, p=0.97 2-tailed Mann-Whitney test).

B: ROC analysis of IL23 LPMC enrichment scores in patients with clinical response at week 8 who had received ustekinumab in UNIFI trial, AUC=0.50.

6.2.6. Stratification of enrichment scores can identify patients who may respond better to ustekinumab

Having been able to demonstrate statistically significant differences in enrichment scores of several outcome measures at week 8 we postulated that by stratifying patients by their enrichment scores we could identify groups of patients who may have better or worse outcomes than if they were unstratified.

Therefore, we divided the patients who had received ustekinumab (n=364) and split then into terciles and calculated the percentage of patients who achieved clinical remission, endoscopic healing, and deep remission.

As shown in figures 6.2.6.1. and 6.2.6.2. by stratifying patients by enrichment score patients can be identified who have a better (or worse) chance of meeting endpoints. For example, deep remission was achieved in 11.4% at week 8 of all patients who received ustekinumab, however if the IL23 LPMC enrichment score was <0 then the chance rises to 15.3% and if the IL23 LPMC enrichment score is >0.28 then the chance of deep remission is only 5.5%.

There is statistically significant reduction in percentage of patients achieving all the endpoints if the enrichment score is >0.28 but no other enrichment scores

had statistically different differences to unstratified patients in all the endpoints. This finding is similar to the findings seen in 6.2.2- 6.2.5, namely that higher IL23 LPMC enrichment scores were associated with non-response. Discussion of this finding can be seen in 7.1.4

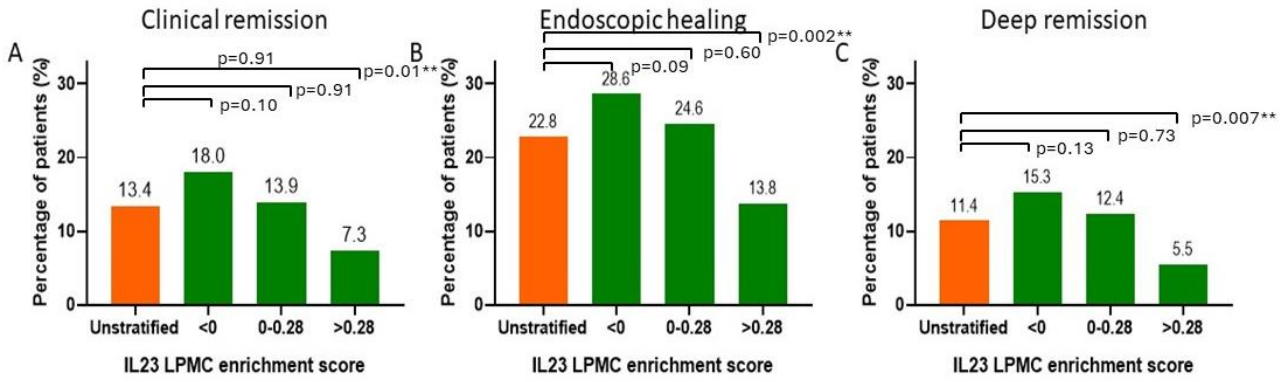


Figure 6.2.6.1. 3 graphs shown percentage of patients who received ustekinumab as part of UNIFI trial and achieved outcome at week 8 stratified by IL23 LPMC enrichment score. Orange column demonstrates percentage of all patients who achieved outcome. Green bars are percentage of patients stratified by IL23 LPMC enrichment score (ES). Comparison of percentages compared to unstratified group using Fisher's exact test.

A – clinical remission: unstratified; 49/364 (13.4%), ES <0; 24/133 (18.0%), p=0.10, ES 0-0.28; 17/122 (13.9%), p=0.91, ES >0.28; 8/109 (7.3%), p=0.01.

B - endoscopic healing: unstratified; 83/364 (22.8%), ES <0; 38/133 (28.6%), p=0.09, ES 0-0.28; 30/122 (24.6%), p=0.60, ES >0.28; 15/109 (13.8%), p=0.002.

C - deep remission: Unstratified 41/361 (11.4%), ES <0; 20/131 (15.3%), p=0.13, ES 0-0.28; 15/121 (12.4%), p=0.73, ES>0.28; 6/109 (5.5%), p=0.007

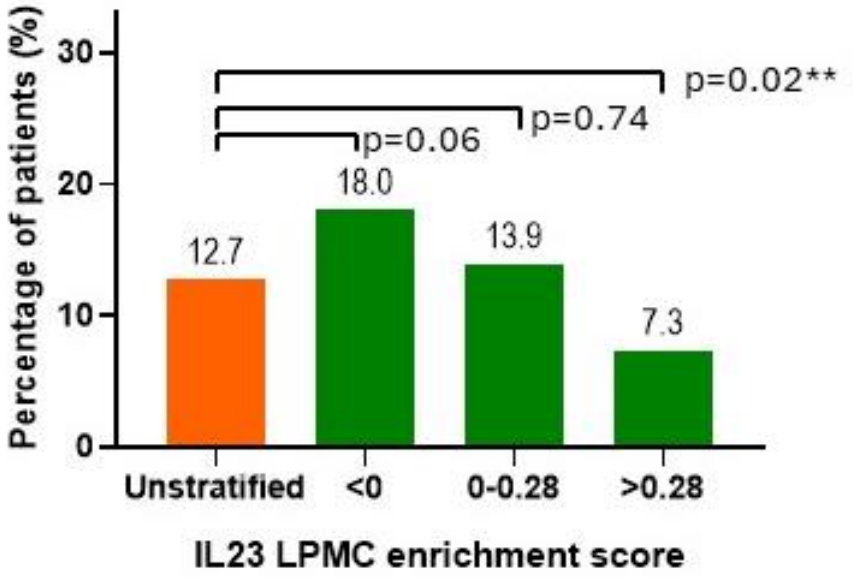


Figure 6.2.6.2. Graph shows percentage of patients who received ustekinumab as part of UNIFI trial and achieved clinical and endoscopic remission at week 8 stratified by IL23 LPMC enrichment scores. Orange column demonstrates percentage of all patients who achieved outcome - unstratified 46/361 (12.7%). Green bars are percentage of patients stratified by IL23 LPMC enrichment score (ES), ES <0 24/133 (18.0%), p=0.06, ES 0-0.28 17/122 (13.9%), p=0.74, ES >0.28 8/109 (7.3%), p=0.02.

Comparison of percentages compared to unstratified group using Fisher's exact test.

6.2.7. Combined enrichment score and biochemical analyses

We had access to biochemical data at week 0 which we postulated may enhance the prediction of response when combined with IL23 LPMC enrichment scores.

When analysing figure 6.2.7. it shows the percentage of patients who have achieved combined clinical and endoscopic remission at week 8. This graph shows greater percentage of patients achieving this endpoint when stratified by IL23 LPMC enrichment score (ES) as well as by CRP. Unstratified patients had 12.7% chance of meeting the endpoint but when ES and CRP are combined it identifies a group of patients with IL23 LPMC enrichment score <0 and CRP <5 with 22.8% of patients achieve this endpoint. Importantly there is also identification of a group of patients with IL23 LPMC enrichment score >0.28 and CRP >5 who are unlikely to achieve the endpoint who may benefit from alternative therapeutic strategies. Statistically significant reduction in the likelihood of meeting clinical and endoscopic remission was seen when comparing unstratified group with patients with CRP>5 as well as when IL23 LPMC enrichment score was > 0.28 and CRP >5.

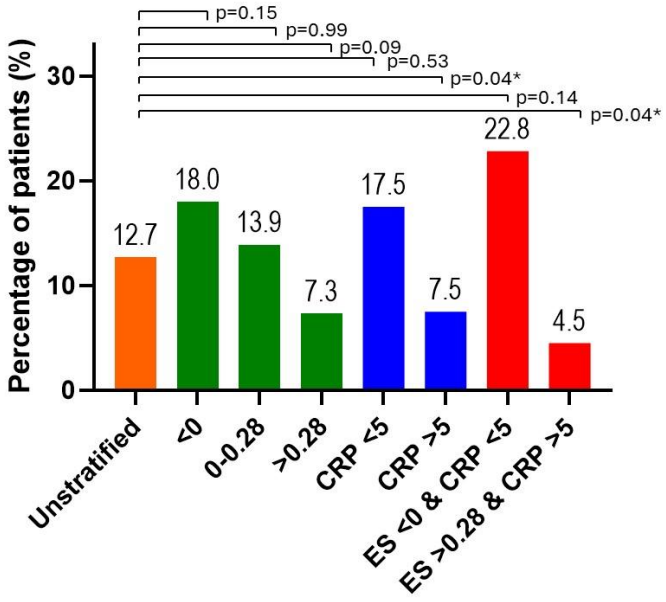


Figure 6.2.7. Graph shows percentage of patients who received ustekinumab as part of UNIFI trial and achieved clinical and endoscopic remission at week 8 stratified by IL23 LPMC enrichment score and week 0 CRP.

Orange column demonstrates percentage of all patients who achieved outcome - unstratified 46/361 (12.7%).

Green bars stratified by IL23 LPMC enrichment score (ES), ES <0 24/133 (18.0%) p=0.15, ES 0-0.28 17/122 (13.9%), p=0.99, ES >0.28 8/109 (7.3%), p=0.09.

Blue bars stratified by CRP. CRP <5 32/183 (17.5%), p=0.53, CRP >5 13/174 (7.5%), p=0.04.

Red bars stratification by ES and CRP. ES <0 & CRP <5 18/79 (22.8%), p=0.14, ES >0.28 & CRP >5 3/67 (4.5%), p=0.04

Comparison of percentages compared to unstratified group using Fisher's exact test.

6.2.8. Analysis of patients receiving placebo reveals a group of patients who may not require any additional therapy

We chose to analyse patients who had received placebo in particular those who responded particularly to an objective endpoint as identification of these patients would be clinically useful as they may not require any change in their therapy and therefore avoid potential side effects and cost.

Initial analysis was to compare the IL23 LPMC enrichment scores of patients who received placebo versus those who received ustekinumab as shown in figure 6.2.8.1. This figure demonstrates that there was no significant difference in enrichment scores which is reassuring for further analyses. It should be noted that there was no significant differences in measured biochemical indices, drug exposure, age of patient, sex, age, weight or length of disease as shown in the published UNIFI trial (Sands, Sandborn, et al. 2019).

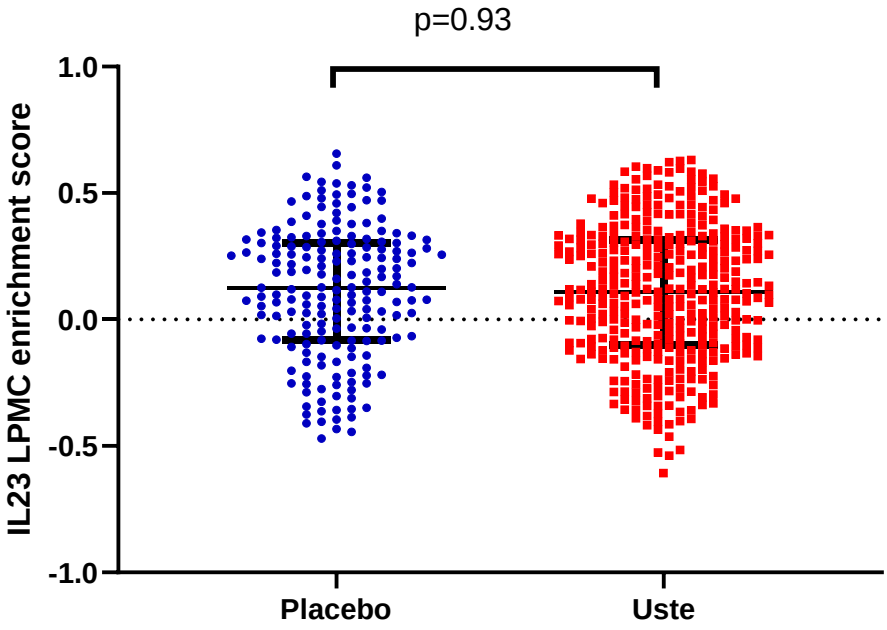


Figure 6.2.8.1. Graph of IL23 LPMC enrichment scores of patients at week 0 in UNIFI trial receiving either placebo or ustekinumab. Placebo: n= 186, median = 0.12, IQR= -0.08 – 0.30; ustekinumab n= 364, median = 0.11, IQR= -0.10 – 0.32; p=0.93 2-tailed Mann-Whitney test.

We then compared IL23 LPMC enrichment scores in those patients who had received placebo and achieved clinical and endoscopic remission versus those who had not as shown in figure 6.2.8.2. which shows a significantly lower IL23 LPMC enrichment score and associated ROC curve with AUC of 0.69.

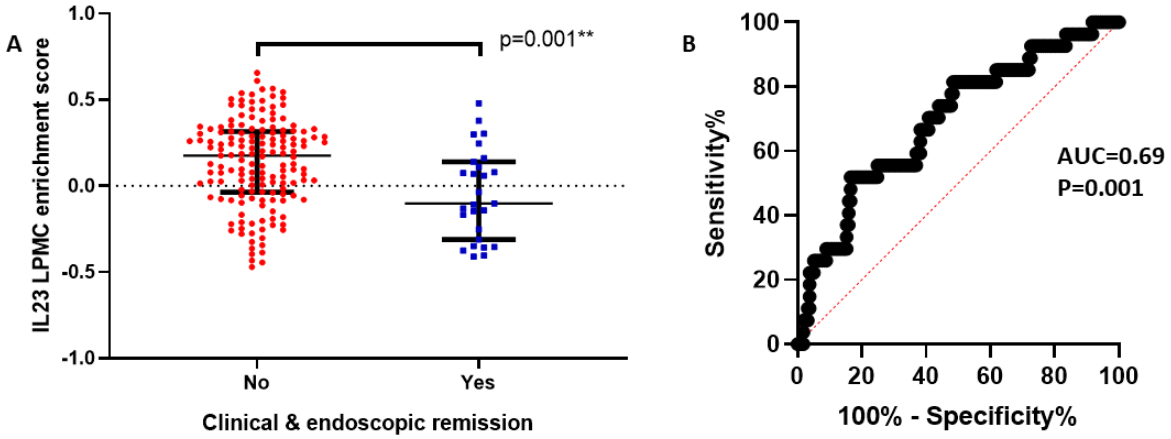


Figure 6.2.8.2. graphs and area under the curve of IL23 LPMC enrichment scores in patients who had not achieved clinical remission and endoscopic healing who had received placebo in UNIFI trial at week 8

A - graph of IL23 LPMC enrichment scores in patients who had an had not achieved clinical and endoscopic remission at week 8 who had received placebo in UNIFI trial at week 8 (yes: n= 27, median=-0.10, IQR=-0.31 – 0.14; no n= 157, median=0.18, IQR=-0.04 – 0.32; p=0.001 2-tailed Mann-Whitney test).

B - ROC analysis of IL23 LPMC enrichment scores in patients with clinical and endoscopic remission at week 8 who had received placebo in UNIFI trial, AUC=0.69.

6.3. Comparison of enrichment scores in anti-TNF responders and non-responders

Having shown statistical differences in enrichment scores in responders and non-responders to IL23p40 blockade we decided to evaluate or IL23 LPMC gene signature against anti-TNF cohorts to see if the changes seen were specific to IL23p40 blockade or had wider applications. We hypothesised that anti-TNF non-responders would have an increased enrichment score compared with anti-TNF responders.

We used the same IL23 LPMC signature used previously (upregulated transcripts with unadjusted $p<0.01$) in two reposited datasets GSE16879 (Arijs, De Hertogh, et al. 2009) and GSE23597 (Toedter et al. 2011) but also in a dataset provided by MSD from the PURSUIT golimumab in UC.

6.3.1. IL23 LPMC gene signature in PURSUIT golimumab dataset

This dataset comprises 152 patients with UC all of whom received golimumab as part of the PURSUIT trial (Sandborn, Feagan, Marano, Zhang, Strauss, Johanns, Adedokun, Guzzo, Colombel, Reinisch, Gibson, Collins, Järnerot, et al. 2014).

MSD used our IL23 LPMC gene signature to produce GSVA enrichment scores and also provided clinical information and clinical trial outcomes.

Whilst there were similar endpoints in the PURSUIT trial as the UNIFI trial there were some differences in the definitions. For example, endoscopic remission was denied as Mayo 0 or 1 in both studies though PURSUIT was at week 6, whereas UNIFI was week 8. It should be noted that the patient populations are different (as well as the pre-defined endpoints) and therefore direct comparison is not possible.

6.3.1.1. IL23 LPMC gene signature positively correlates with biochemical but not demographic measures of ulcerative colitis

Firstly, we wanted to see if the enrichment scores correlated with clinical and biochemical markers. We hypothesised that it would not correlate as the enrichment score should not be related to severity of disease (CRP, calprotectin or lactoferrin) nor should it be related to duration of disease or Mayo score.

As shown in figure 6.3.1.1. it shows 5 graphs showing correlation of IL23 LPMC enrichment score to CRP, faecal calprotectin and faecal lactoferrin, total Mayo score at baseline and duration of disease. Linear regression is extremely weak

for all measured values though faecal calprotectin, CRP and total Mayo score show statistically significant positive correlation.

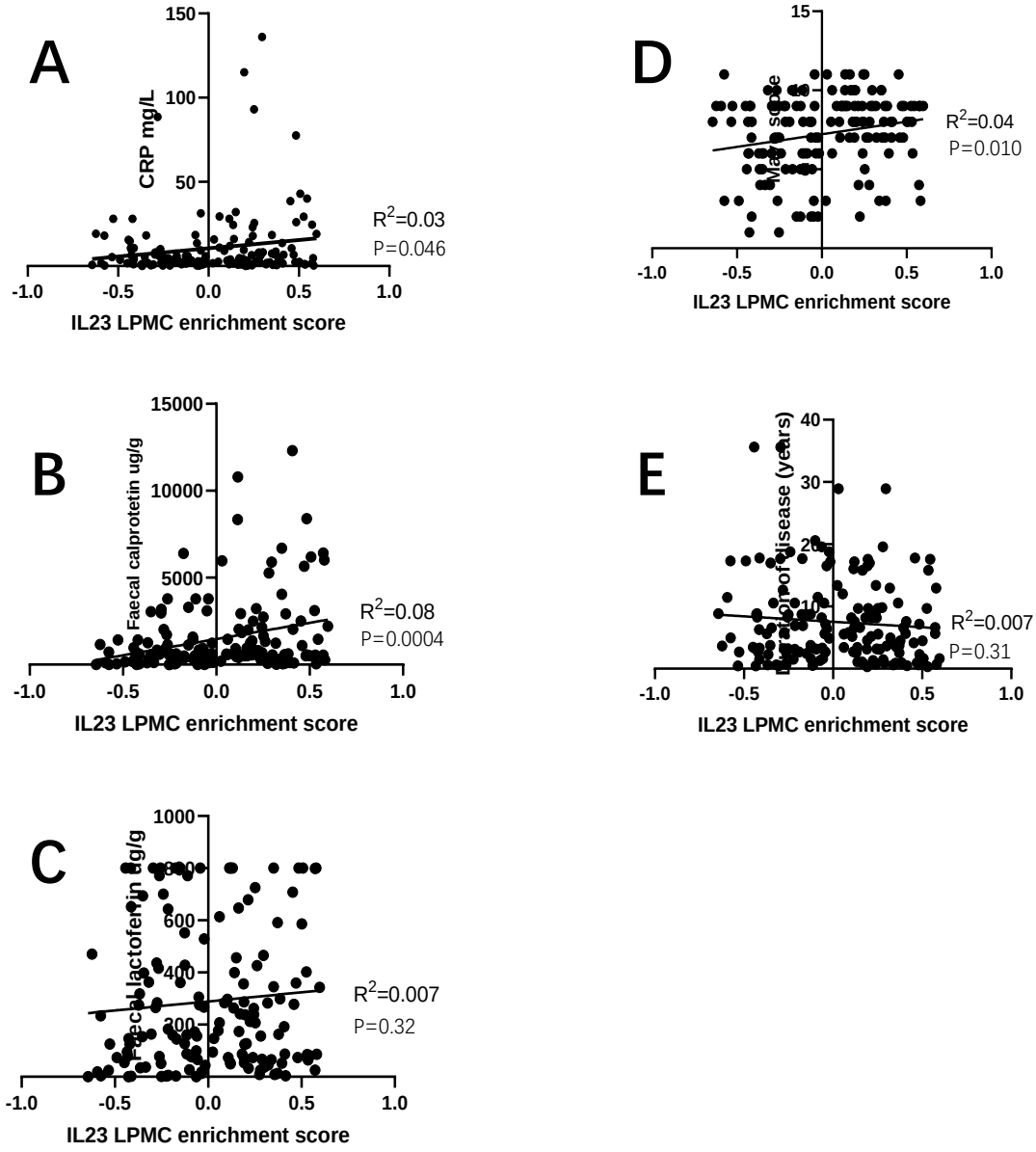


Figure 6.3.1.1. showing graphs plotting IL23 LPMC enrichment scores of each sample with biochemical values of: A- CRP R^2=0.03, p=0.046, B - faecal calprotectin R^2= 0.08, p=0.0004, C - faecal lactoferrin R^2=0.007, p=0.32, D - total Mayo score R^2= 0.04, p=0.01 and E - duration of disease R^2= 0.007, p=0.31. Linear regression is shown as goodness of fit R^2

6.3.1.2. IL23 LPMC gene signature predicts endoscopic mucosal healing at week 6

Endoscopic mucosal healing (endoscopic Mayo score of 0 or 1) at week 6 was plotted against IL23 LPMC enrichment score as seen in figure 6.3.1.2.1. This shows statistical difference between the endoscopic mucosal healing responders and non-responders (p=0.02). The non-responders have a higher enrichment score than the responders which agrees with our hypothesis.

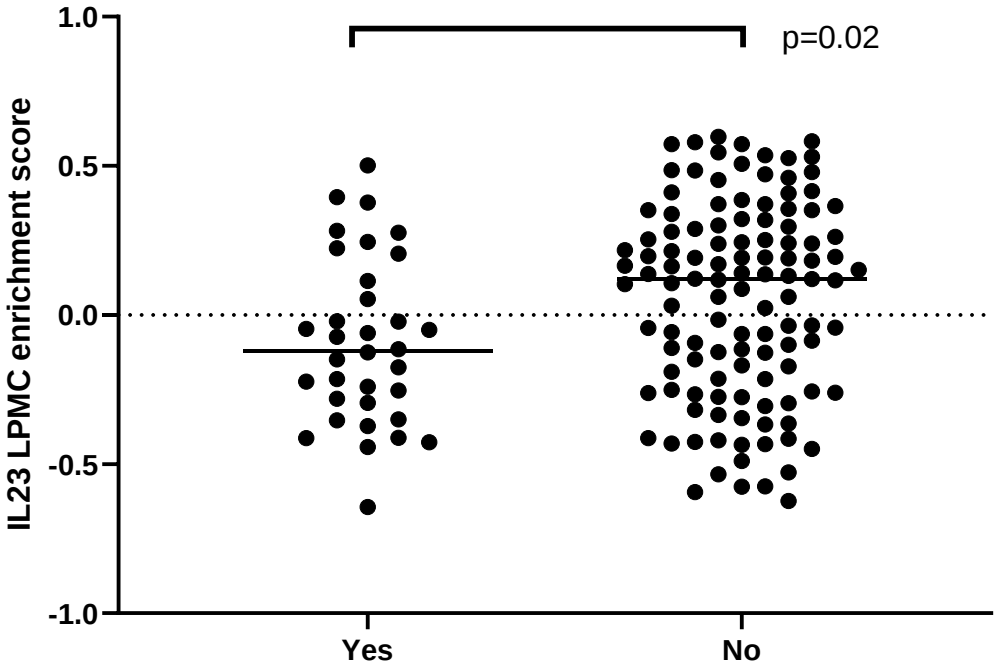


Figure 6.3.1.2.1 graph of IL23 LPMC enrichment scores in patients with ulcerative colitis who have and have not achieved endoscopic remission at week 6 to golimumab in PURSUIT trial. Responders: n=34, median=-0.12, IQR=-0.31 – 0.14, non-responders: n= 118, median=0.12, IQR=-0.22 – 0.31. p=0.02 2-tailed unpaired t-test.

Using the same data of IL23 LPMC enrichment scores of patients achieving endoscopic remission in the PUSUIT trial AUC 0.63 (95% CI 0.53-0.73) as demonstrated in figure 6.3.1.2.2.

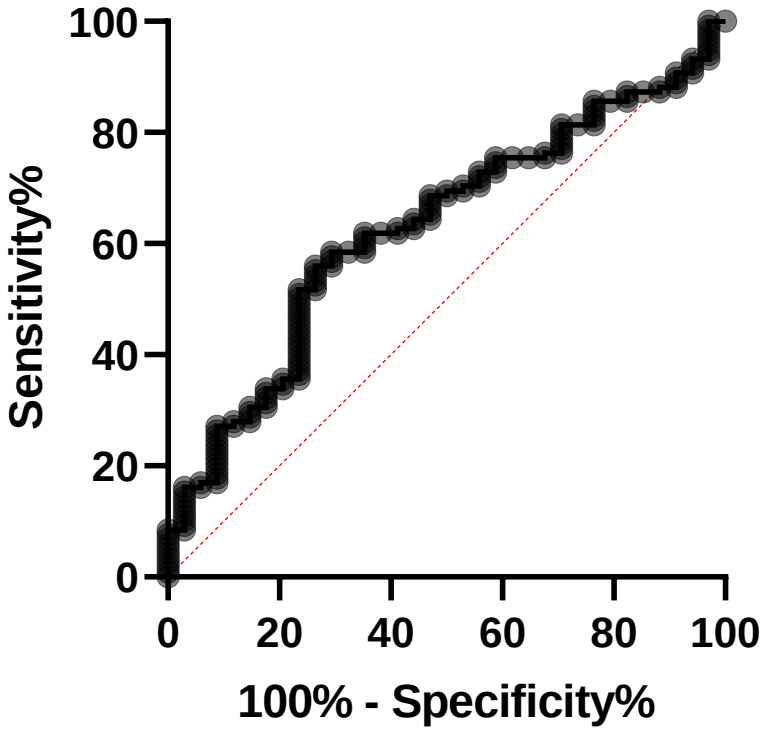


Figure 6.3.1.2.2. Receiver operator curve (ROC) curve of IL23 LPMC enrichment scores in patients who have and have not achieved endoscopic remission at week 6 in PURSUIT trial of golimumab in patients with ulcerative colitis. Area under curve (AUC) 0.63, 95% CI 0.53-0.73.

6.3.1.3. IL23 LPMC gene signature does not predict clinical response at week 6 in the PURSUIT trial

Next, we evaluated whether the IL23 LPMC gene signature could be used to separate responders and non-responders to golimumab. We compared the enrichment scores of the patients who did and did not achieve these endpoints.

Clinical response (decrease of total Mayo score ⩾ 30% and ⩾3) was plotted against IL23 LPMC enrichment score as seen in figure 6.3.1.3. This shows no statistical difference between the clinical responders and non-responders (p=0.08).

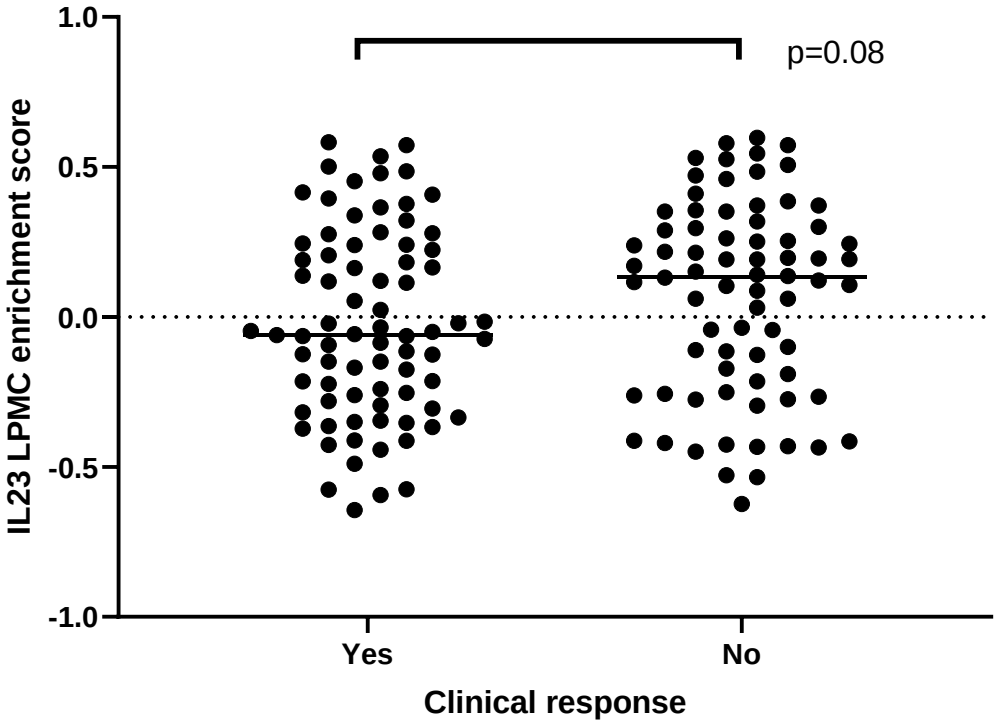


Figure 6.3.1.3. graph of IL23 LPMC enrichment scores in patients with ulcerative colitis who have and have not achieved clinical response at week 6 to golimumab in PURSUIT trial. n=152, clinical responders: n=78, median=-0.06, IQR=-0.28 – 0.24, clinical non-responders: n=74, median=0.13, IQR=-0.22 – 0.31. p=0.08, 2-tailed unpaired t-test.

6.3.1.4. IL23 LPMC gene signature does not predict clinical remission at week 6

Clinical remission (total Mayo score ⩽ 2) at week 6 was plotted against IL23 LPMC enrichment score as seen in figure 6.3.1.4. This shows no statistical difference between the clinical responders and non-responders (p=0.10).

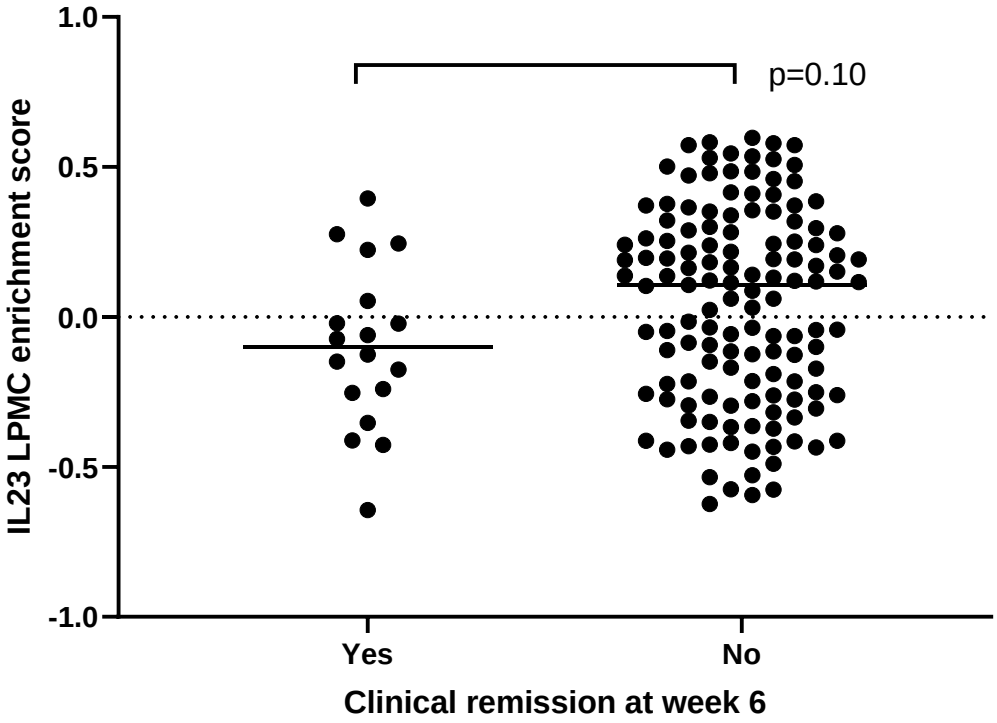


Figure 6.3.1.4. graph of IL23 LPMC enrichment scores in patients with ulcerative colitis who have and have not achieved clinical remission at week 6 to golimumab in PURSUIT trial. Clinical responders: n=18, median=-0.10, IQR=-0.28 – 0.10, clinical non-responders: n= 134, median=0.11, IQR=-0.26 – 0.29. p=0.10 2-tailed unpaired t-test.

6.3.1.5. Stratification of enrichment scores can improve likelihood of endoscopic remission in PURSUIT trial

A further method to analyse this data is to compare the endpoint achievement rate to that of patients with an IL23 LPMC enrichment score in a specific range. As shown below in figure 6.3.1.5.1 which shows in blue the percentage of patients who achieved the target from the whole trial (unstratified). Adjacent to this in red are the percentage of patients achieving this target stratified to enrichment scores. As can be seen in all 3 graphs it is possible to improve the likelihood of achieving these endpoints by selecting patients with a specific enrichment score however none of the analyses show a significant statistical difference compared to the unstratified group.

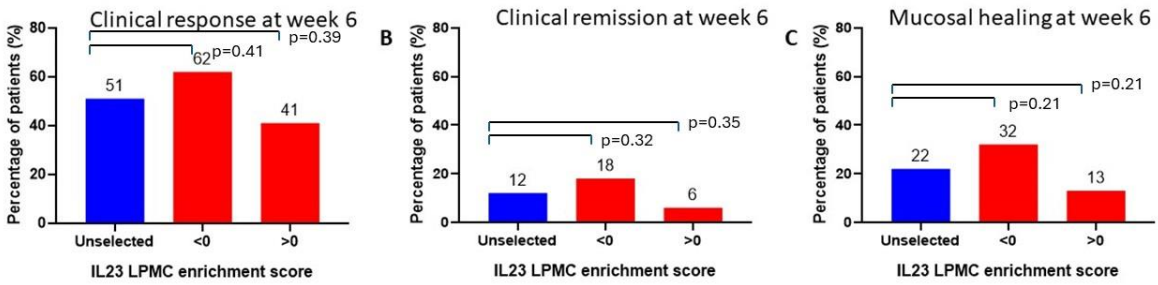


Figure 6.3.1.5.1. 3 graphs show percentage of patients who achieved endpoints in PURSUIT trial of golimumab in UC. Blue bars represent the percentage of patients in the whole trial (n=152) whereas the red bars stratify patients by IL23 LPMC enrichment scores.

Graph A clinical response at week 6, unselected (78/152, 51%) enrichment score <0 (46/74, 62.2%), p=0.41 and enrichment score >0 (32/78, 41.0%), p=0.39

Graph B – clinical remission at week 6, unselected (18/152, 12%) enrichment score <0 (13/74, 18%), p=0.32 and enrichment score >0 (5/78, 6%), p=0.35

Graph C – mucosal healing at week 6, unselected (33/152), enrichment score <0 (24/74, 32%), p=0.21 and enrichment score >0 (10/78, 13%), p=0.21

Comparison of percentages compared to unstratified group using Fisher's exact test.

Furthermore figure 6.3.1.5.2. shows a similar graph with clinical and endoscopic remission which shows non statistically significant changes.

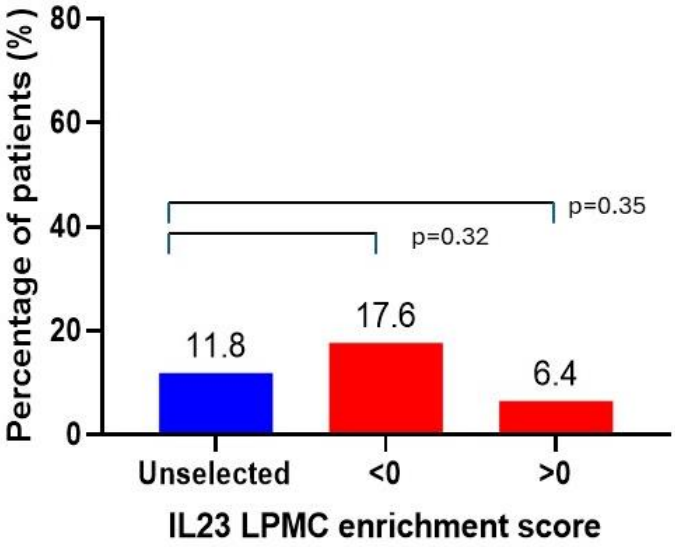


Figure 6.3.1.5.2. Graph show percentage of patients who achieved clinical remission and endoscopic healing endpoint at week 6 in PURSUIT trial of golimumab in UC. Blue bars represent the percentage of patients in the whole trial (18/152=11.8%) whereas the red bars stratify patients by IL23 LPMC enrichment scores.

<0: 13/74 (17.6%), p=0.32

>0: 5/78 (6.4%), p=0.35.

Comparison of percentages compared to unselected group using Fisher's exact test.

6.3.1.6. Combination of enrichment score and biochemical markers can improve prediction of response to golimumab

Analysing the data further highlighted the potential that CRP at baseline could be used to discriminate between responders and non-responders. For example, when analysing clinical response at week 6 a CRP<5 56/84 (66.7%) patients responded vs 22/66 if CRP >5 (33.3%).

Therefore, we analysed the data further to see if a combined enrichment score and biochemical marker can be used to better predict response to golimumab than just enrichment score or CRP alone.

The results of analysing CRP and IL23 LPMC enrichment scores are summarised below in figures 6.3.1.6.1, figure 6.3.1.6.2., 6.3.1.6.3., 6.3.1.6.4. All the endpoints analysed (clinical response, clinical remission, mucosal healing and combined mucosal healing and clinical remission) show the percentage of patients achieving it increased by using CRP and ES together however none of these observations are statistically significant.

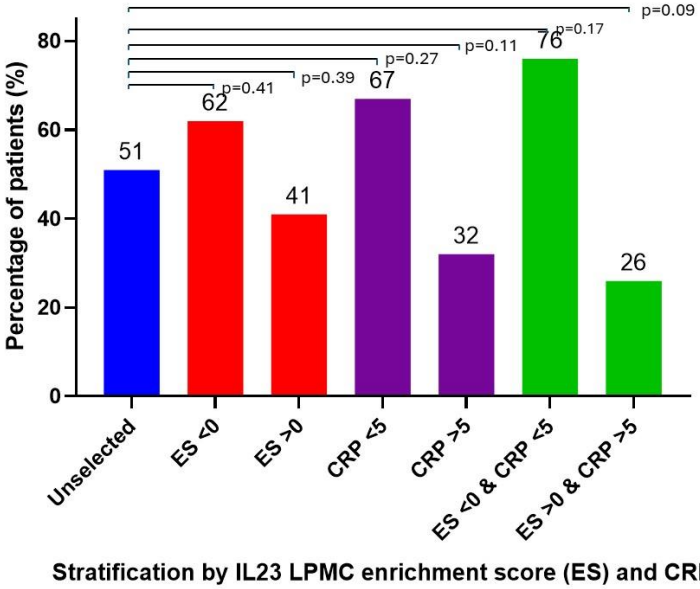


Figure 6.3.1.6.1. Graph showing percentage of patients achieving clinical response at week 6 in PURSUIT trial of golimumab in UC stratified by IL23 LPMC enrichment score and CRP. Blue bar is all patients (78/152, 51.3%)

Red bars indicating stratification by IL23 LPMC enrichment score <0 (46/74, 62.2%), 0.41, and enrichment score >0 (32/78, 41.0%), p=0.39.

Purple bars stratification by baseline CRP <5 (56/84, 66.7%), p=0.27, and CRP >5 (22/68, 32.3%), p=0.11

Green bars show stratification by combined enrichment score <0 & CRP <5 (34/45, 75.6%), p=0.17, and enrichment score >0 and CRP>5 (10/39, 25.6%), p=0.09.

Comparison of percentages compared to unselected group using Fisher's exact test.

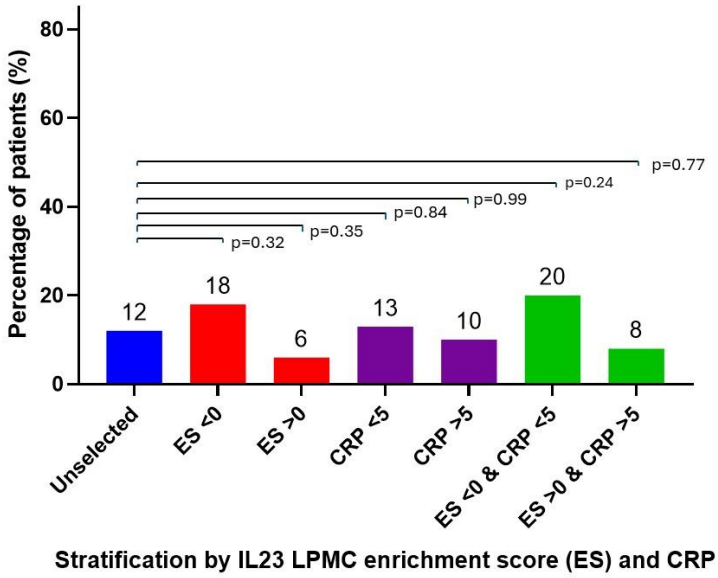


Figure 6.3.1.6.2. Graph showing percentage of patients achieving clinical remission at week 6 in PURSUIT trial of golimumab in UC stratified by IL23 LPMC enrichment score and CRP. Blue bar is all patients (18/152, 11.8%)

Red bars indicating stratification by IL23 LPMC enrichment score <0 (13/74, 17.6%), p=0.32 and enrichment score >0 (5/78, 6.4%), p=0.35.

Purple bars stratification by baseline CRP <5 (11/84, 13.1%), p=0.84 and CRP >5 (7/68, 10.3%), p=0.99

Green bars show stratification by combined enrichment score <0 & CRP <5 (9/45, 20.0%), p=0.24, and enrichment score >0 and CRP>5 (3/39, 7.7%), p=0.77

Comparison of percentages compared to unselected group using Fisher's exact test.

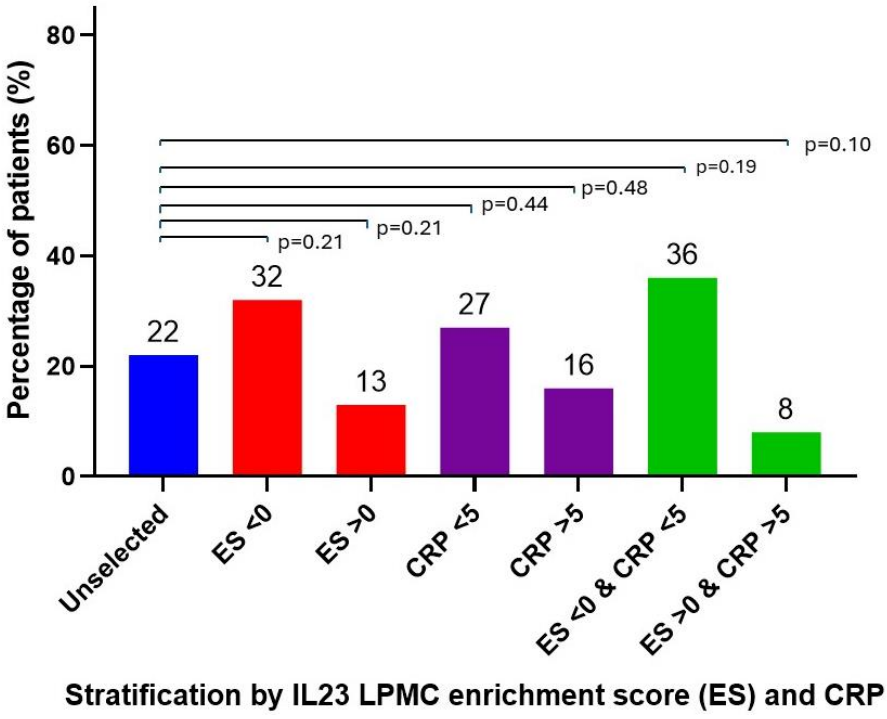


Figure 6.3.1.6.3. Graph showing percentage of patients achieving mucosal healing at week 6 in PURSUIT trial of golimumab in UC stratified by IL23 LPMC enrichment score and CRP. Blue bar is all patients (34/152, 22.3%)

Red bars indicating stratification by IL23 LPMC enrichment score <0 (24/74, 32.4%), p=0.21, and enrichment score >0 (10/78, 12.8%), p=0.21.

Purple bars stratification by baseline CRP <5 (23/84, 27.4%), p=0.44 and CRP >5 (11/68, 16.2%), p=0.48

Green bars show stratification by combined enrichment score <0 & CRP <5 (16/45, 35.6%), p=0.19 and enrichment score >0 and CRP>5 (3/39, 7.7%), p=0.10.

Comparison of percentages compared to unselected group using Fisher's exact test.

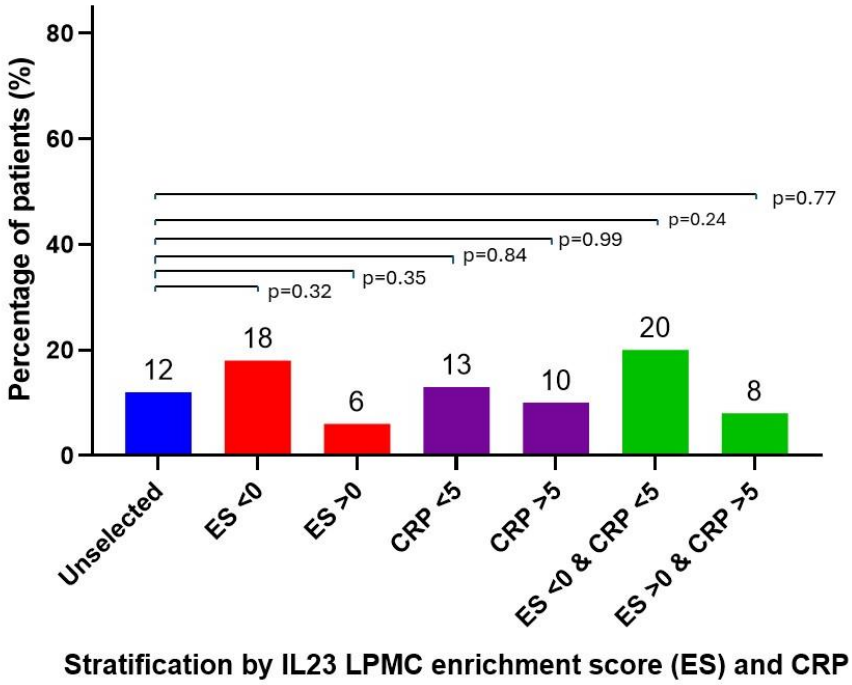


Figure 6.3.1.6.4. Graph showing percentage of patients achieving clinical and mucosal healing at week 6 in PURSUIT trial of golimumab in ulcerative colitis stratified by IL23 LPMC enrichment score and CRP. Blue bar is all patients (18/152, 11.8%)

Red bars indicating stratification by IL23 LPMC enrichment score <0 (13/74, 17.6%), p=0.32 and enrichment score >0 (5/78, 6.4%), p=0.35.

Purple bars stratification by baseline CRP <5 (11/84, 13.1%), p=0.84 and CRP >5 (7/68, 10.3%), p=0.99.

Green bars show stratification by combined enrichment score <0 & CRP <5 (9/45, 20.0%), p=0.24 and enrichment score >0 and CRP>5 (3/39, 7.7%), p=0.77

Comparison of percentages compared to unselected group using Fisher's exact test.

6.3.2. No statistical difference in enrichment scores between infliximab responders and non-responders in dataset GSE16879 using IL23 LPMC gene signature

As can be seen in figure 6.3.2. there is no statistical difference in GSVA enrichment scores between responders and non-responders of infliximab GSE16879 defined responders as endoscopic and histological healing at week 8.

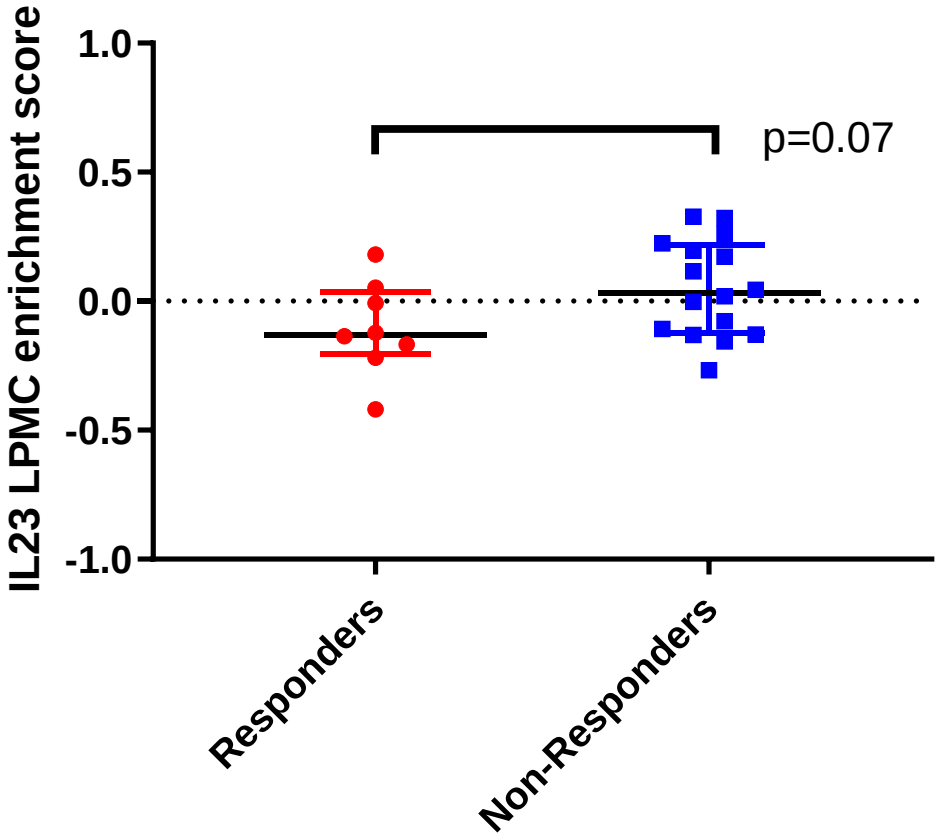


Figure 6.3.2. graph of gene set variation analysis (GSVA) score using reposited gene set GSE16879 showing Responders (to infliximab) and Non-responders (to infliximab) using gene list generated from upregulated differentially expressed genes with p<0.01 from lamina propria mononuclear cells from patients with active ulcerative colitis cultured with standard media +/- IL-23. Responders (n=8, median = -0.13, IQR: -0.21 – 0.04) and non-responders (n=16, median= 0.03, IQR: -0.12 – 0.22). No statistical difference in GSVA enrichment was seen between infliximab responders and non-responders (p=0.07 Mann-Whitney test, two tailed). Data displayed with median and interquartile range bars.

6.3.3. No statistical difference in enrichment scores between infliximab responders and non-responders in dataset GSE23597 using IL23 LPMC gene signature

We calculated GSVA enrichment scores for reposited dataset GSE23597. There was no significant difference in enrichment. GSE23597 defined responder as decrease in total Mayo score ⩾ 3 (with 1 point reduction in endoscopic Mayo score).

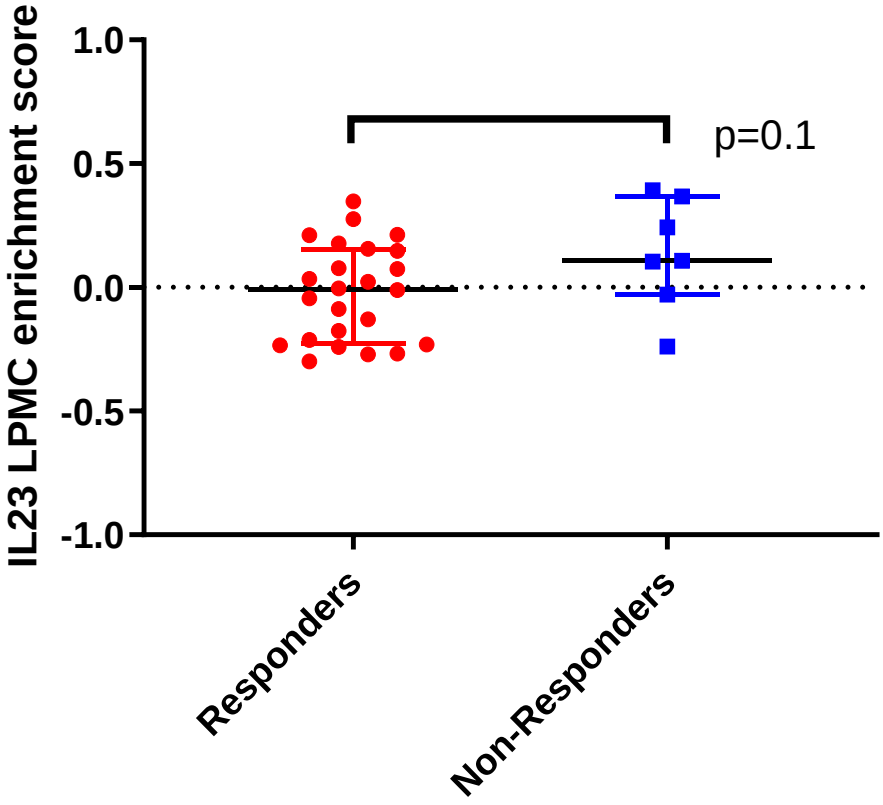


Figure 6.3.3. graph of gene set variation analysis (GSVA) score using reposited gene set GSE23597 showing Responders (to infliximab) and Non-responders (to infliximab) using gene list generated from upregulated differentially expressed genes with p<0.01 from lamina propria mononuclear cells from patients with active ulcerative colitis cultured with standard media +/- IL-23. Responders (n=24, median = 0.01, IQR: -0.23 – 0.15) and Non-responders (n=7, median= 0.11, IQR: -0.03 – 0.37). No statistical difference in enrichment score was seen between infliximab responders and non-responders (p=0.10 Mann-Whitney test, two tailed). Data displayed with median and interquartile range bars.

6.4. Conclusion

Using our transcription-based signatures has had mixed results. We have demonstrated with the IL23 LPMC derived signature that it is highly expressed in active UC compared to healthy controls. Moreover, we have shown the IL23 LPMC derived signature to be overexpressed in patients who do not achieve endoscopic remission at week 8 to anti-TNF in a large clinical trial using golimumab in line with our hypothesis.

Furthermore, we demonstrated that our IL23 LPMC signature was differentially expressed in patients who responded to ustekinumab in UNIFI trial. Intriguingly however the IL23 LPMC signature was overexpressed in patients who did not respond to ustekinumab which was contrary to our hypothesis.

Furthermore, we established that using a combination of enrichment scores as well as CRP can increase the predictive value which would improve clinical utility.

7. Conclusion

7.1. Discussion

7.1.1. IL-23 stimulating of LPMCs strongly induces Th17 pathway response

IL-23 induced a statistically significant expression in over 200 genes encoding cytokines, chemokines, growth factors, transmembrane receptors, transcription factors, ion channels and enzymes. Whilst predicted downstream effects of IL-22 and other Th17 genes, also encoded were other less expected genes most notably IFN-γ as discussed below.

Furthermore, upstream regulator analysis showed highly relevant IL23A and IL12B, other predicted activators included IL1 pathway components (IL1A, IL1B, IL1R, MYD88), Toll-like receptor agonists (LPS, imiquimod) and other inflammatory cytokines (IFNG, IL21, IL6, IL33, TNF).

Overall, this provides great reassurance that IL-23 has stimulated the LPMCs with expected response but furthermore, has revealed other avenues for further exploration such as the link with IL-1 pathway and IFN-γ.

7.1.2. Interferon-γ was induced by IL-23 in LPMC experiments

Interferon-γ was one of the most statistically significantly induced cytokines when LPMCs were stimulated with IL-23 (Chapter 4). As a Th1 cytokine this was a surprise as we expected to see Th17 cytokines almost exclusively.

IFNγ is canonically produced by CD4 cells when stimulated by IL-12 and is the signature Th-1 cytokine (Monteleone et al. 1997{Parronchi, 1997 #314)}(Ito et al. 2006). However, though that paradigm appears to be changing with a more complex with a number of examples of IFNγ in response to IL-23 which speaks of a Th-1 / Th-17 plasticity (Liu et al. 2011; Ziblat et al. 2018). IL-23 signalling can drive the conversion of Th17 to Th1 cells by shifting the secretion of IL17A to IFNγ in vivo (Hirota et al. 2011). Moreover, IL-23 may suppress IL-17 expression and enhance IFNγ release through a STAT4/T-bet-dependent pathway, particularly under conditions of decreased TGFβ expression (Ueno et al. 2015). Furthermore, a murine model with CD4+ T cells lacking the IL23R has revealed that IL23R signalling induces colitis, associated with the induction of IFNγ and IL17A co-expressing cells (Ahern et al. 2010).

This unexpected result speaks of the complex interplay of cytokines and CD4 subsets of which we are still expanding our knowledge. The 'simple' paradigm of Th1 and Th2 mediated cytokines is no longer valid as there is significant crosstalk of cytokines between cell types. Whilst our experiments do not provide all the answers regarding the interaction between IL-23 and IFNγ production it does serve to highlight the gaps of knowledge that still exist in our understanding of cytokine interplay in IBD.

7.1.3. Anti-IL12p40 had higher response to endpoints in patients with lower enrichment score

In chapter 6 we analysed the enrichment of the IL-23 LPMC signature in a reposited dataset from the UNIFI trial. We had hypothesised that patients who would achieve the given endpoint (e.g., clinical remission, endoscopic remission etc) would have a higher enrichment score than those who did not achieve that endpoint. However, we found the opposite contrary to our initial hypothesis.

At first this appeared hard to explain. We expected the samples with higher expression of IL-23 induced genes to have a better response to a drug which targets the IL-23 receptor. One explanation may be due to multiple

activated inflammatory pathways in patients who did not respond with significant overlap of the genes involved (e.g., STAT3, DOK5, FGF7). Therefore, the IL-12/IL-23 pathway may not have been dominant and so its blockade has not had the dramatic effect which was proposed.

A second reason is the effect of the blockade of the IL-12 pathway may alter or reduce the anti-inflammatory effect of the IL-23 blockade and hence why the more IL23 enriched samples did not meet the endpoints we expected. Our IL-23 LPMC signature comprises of genes which have been upregulated by IL-23 only whereas antiIL12p40 drugs also block IL-12 receptor. The effect of blocking IL-12 and IL-23 concurrently compared to IL-23 alone is unknown. Whilst caution should be heeded when comparing IBD to other diseases, in psoriasis anti-IL23p19 drugs have been shown to be superior to anti-IL12p40 (Papp et al. 2017). The implication behind this statement may be that the blockade of IL-12 may have a regulatory effect such that IBD does not ameliorate as much as with IL-23 blockade alone. Therefore, if this experiment was repeated with reposited data from anti-IL23p19 trial patients in future using the same IL23 LPMC signature I suspect that we may see a different pattern of enrichment scores in responders v non-responders.

Finally, the responders may have been patients with less severe disease who may have had less intensity of IL23 responsive genes. Whereas the patients with more severe disease who had a higher enrichment of IL23 responsive genes may not have reached the endpoint. A hint of this is seen in that clinical responders had no difference in enrichment scores and that patients with a lower CRP had a higher chance of achieving clinical endpoints. It is feasible that the more severe patients with higher IL23 gene enrichment required a longer course of treatment to affect endoscopic and histological change. Unfortunately, we did not have access to data of later endpoints at (week 48 for example) but it would have been interesting to see if the IL23 gene signatures were enriched in endoscopic and histological responders.

7.1.4. IL23 LPMC enrichment scores were significantly higher in patients not meeting UNIFI and PURSUIT endpoints

An interesting finding was that the enrichment scores were higher in those patients who did not meet the defined clinical and endoscopic endpoints in the UNIFI and PURSUIT trials. It may be expected that those patients with a higher IL23 enrichment score would respond better to therapy directed against the stimulus. However, the opposite seemed to be true. This may be explained by the positive correlation between IL23 LPMC enrichment scores and total Mayo

score, CRP, lactoferrin and calprotectin which are markers of active disease. With worsening inflammation there may be an increased IL-23 induced inflammatory cascade and hence overexpression of IL-23 induced genes. Therefore, the IL23 LPMC signature may be a marker of active disease rather than having specificity to IL23 responsiveness.

7.1.4. Lower CRP predicts response to golimumab and ustekinumab in UC

It was an interesting observation that in both industry sponsored trial data (PURSUIT and UNIFI) that lower CRP predicted response to therapy. In CD a higher CRP has been associated with improved response to therapies (Sandborn et al. 2007; Colombel et al. 2010; Reinisch et al. 2012) though in UC this finding has not been replicated using baseline CRP.

Explanations for this finding are unclear but may be that patients who had less severe disease had a lower CRP and therefore had a better response to therapy. Alternative hypotheses may be due to altered pharmacokinetics of the drug due to higher disease burden and a lower albumin leading to lower (and subtherapeutic) drug levels. We were not permitted access to the endoscopic disease severity scores, albumin, or drug level data to explore this hypothesis further but it would be interesting to explore further.

7.1.5. IL23 LPMC gene signature shows statistically significant difference in enrichment scores in PURSUIT trial for objective but not subjective endpoints

It is interesting that the only target which we found to have a statistically significant ability to predict response beyond chance was the only measure which was objective. Both clinical response and clinical remission are either entirely or heavily weighted towards patient reported outcomes which often do not relate to the degree of disease present. The only objective marker in our dataset is of endoscopic healing and so it is pleasing that our IL23 LPMC gene signature can separate the responders and non-responders by their enrichment scores.

Interestingly, a similar finding was seen when investigating the phase 2a trial of golimumab in UC using a gene signature created by Arijs et al(Arijs, Li, et al. 2009) (Telesco et al. 2018). In this study the only significant difference in enrichment scores using this gene signature was seen in endoscopic healing and not with clinical response or remission.

7.1.6. Placebo treated patients had a higher than expected healing rate in UNIFI trial

It was an interesting and unexpected finding that there was a subgroup of patients who had received placebo as part of the UNIFI trial had achieved clinical and endoscopic remission, a highly challenging endpoint. Ulcerative colitis can follow a relapsing and remitting pattern and it is possible that the disease spontaneously went into endoscopic remission. Further explanations are a deterioration of symptoms and endoscopic findings by infection. Of more interest would be the long term follow up of patients without the addition of medication or intervention to see how many go into spontaneous remission however this would be highly unethical as a study and therefore this type of data is very unlikely to be forthcoming in the near or distant future.

Detailed data of these patients was not available, but it would be interesting to evaluate the diagnosis of UC (as some may have been misdiagnosed), nature of the medication that they had failed or not tolerated as well as length and severity of disease as there may be some clues as to why they responded so well to placebo.

This interesting finding of a group of favourable disease should be evaluated in other trials as identification of these patients would lead to less treatment and so fewer side effects and less cost.

7.1.7. Length of cytokine stimulation time and dose of explants and LPMC experiments

Before performing the experiments, we debated amongst ourselves the optimal length of time to stimulate the explants and LPMCs for.

On one hand we wanted to capture early RNA signals before there was saturation of the IL-23 receptors and so reduction in the downstream effect of the cytokine. We recognised that unlike with measuring of a protein i.e., the end of the process we did not want too long a stimulation. Furthermore, we recognised that the tissue was likely to progressively die and that this process would lead to significant degradation of RNA which may obscure the true picture of what we were hoping to capture. On the other hand, we did not want too short a stimulation as we may not see an effect of the cytokine upon the transcriptome.

Similar work where LPMCs and colonic explants were stimulated with cytokines (Monteleone et al. 1999; Vossenkamper et al. 2014; Rovedatti et al. 2009) stimulated for 48 hours but the results were proteomic based rather than transcriptomic in their output and we felt this to be too long.

Subsequent work has shown that our stimulation may have been too long. A recent paper showed that stimulation of human macrophages with IL-23 showed that IL-23R cell surface presentation was increased within 15 minutes and that downstream RNA such as JAK1, JAK2, JAK3 and TYK2 can be induced with 1 hour of stimulation (Sun, Hedl, and Abraham 2019).

The optimal length of stimulation using LPMCs is still to be determined and is likely to be dependent on multiple factors including the cytokine which is used as the stimulant. The optimal time of stimulation could be determined using multiple experiments with stimulation at multiple time points though the time, money, and resources that this would involve would likely be prohibitive.

Furthermore, the dose of IL-23 which we stimulated was also debated. We based the decision on the manufacturer's recommendation and prior work in

the laboratory stimulating but it is feasible that other doses may have been optimal.

7.1.8. Explant experiments had only modest and non-canonical effects on gene expression and failed to predict response to therapies

In chapter 4 we analysed the effect of IL-23 upon whole explants taken from patients with UC. Overall, the effects were modest and largely disappointing. The expected activation of the Th17 pathway was not seen and non-canonical pathways were activated with a variety of pro and anti-inflammatory mechanisms. Unfortunately, little useful insights can be gleaned from the experiments.

There are several reasons why the differentially expressed gene signature may have created a different profile to that we expected. A colonic pinch biopsy is a collection of tissue which comprises epithelial cells, lamina propria as well as blood vessels. Most of the literature has focused on the effect of IL-23 upon inflammatory cells which reside in the lamina propria but less has been studied on the effect of IL-23 on the epithelial layer and other tissues though there are some studies suggesting that IL23R expression in the epithelial layer provides a protective effect (Aden et al. 2016). Therefore, any Th17 mediated pro-

inflammatory effect from inflammatory cells in the lamina propria may have become 'clouded' by the effect of IL-23 upon other tissues. This may include regulatory, anti-inflammatory effects which may negate the pro-inflammatory Th17 effects that were seen in the LPMC experiments.

Furthermore, the biopsy was cultured with soluble IL-23 for 4 hours though we did not evaluate the ability of IL-23 to permeate into the biopsy and therefore it is possible that IL-23 did not reach a region of the immune cell laden lamina propria. We chose a 4 hour stimulation after a group discussion, but this was based on expert opinion rather than experimental data. Previous experiments by Monteleone *et al* (Monteleone et al. 1999) and Rovedatti *et al* (Rovedatti et al. 2009) used whole colonic biopsies and exposed them to cytokines but they did not measure transcriptomics but used histology to measure degree of necrosis and also had a longer incubation period (24 hours v 4 hours). Together these points may mean that the immune cells which we thought would produce the most pro-inflammatory cytokines may not have been stimulated in sufficient concentration and for sufficient time to produce a measurable transcriptomic response.

Finally, as a technical point it was appreciated that the RNAseq was performed on multiple lanes. Whilst likely to be only a minor variance in the

results we now appreciate the potential benefits of performing RNAseq in the same lane.

7.1.9 LPMC IL-23 enrichments scores were higher in non-responders

7.2 Limitations

7.2.1. Experimental models not representative of in vivo conditions

Experimental models are not similar to *in vivo* conditions, but every effort was made to reduce these differences where possible. We used well established techniques that mimic *in vivo* conditions using cell culture media and temperature controlled incubators.

The biopsy explant technique has limitations in that there is an assumption that biopsies taken from the same area from the same patient will be physically and metabolically 'identical' which cannot be true. Whilst UC is a continuous inflammation throughout the affected segment of colon, there will be areas of increased and decreased activity within a region so the biopsies will not have exactly the same amount of inflammation and will

therefore have slightly different biological activity. Secondly in the explant experiments the shape and size of the biopsies will differ slightly and so will affect the penetrance of the cytokines to the lamina propria with the resulting alteration to the transcriptional profile. Thirdly the structural composition of the biopsy will vary meaning that biopsies will have more or less laminar propria with its differing cell types to that of the epithelial layer hence leading to potentially different transcriptional change.

Whilst every effort was made to lessen these variables which have the potential to influence transcriptional change it is inevitable our models will not truly represent the effect of cytokines upon tissue in the colon.

Alternative models could be considered if this experiment were repeated such as organoids which could be manipulated into colon shapes exposed to a faecal stream and cytokines could be presented to the cells in a more controlled manner. However, this would remove much of the acute inflammatory cells which are present in the freshly taken biopsies.

7.2.2. Small number of samples sent for RNAseq

The total number of samples that were sent for RNAseq was small (explant n=5, LPMC n=6) which reflects the challenge of obtaining suitable patients for studies, the difficult experimental techniques and expense of RNAseq. By far, working to a stringent budget was the largest limiting factor. Therefore, the transcriptional signatures that were developed assumed that these samples were representative of the entire UC population which is unlikely to be the case. As these experiments were done as a proof of concept study, we now know that the experimental techniques used are robust (especially LPMC) and meaningful so in future experiments the 'n' number can be increased, and the number of colonic biopsy sites could be increased to try to improve the representation of the whole UC population. Power calculations were not performed as the effect size was unknown and this provides a limitation of any significant findings seen.

7.2.3 No histological evaluation to assess for inflammation

To assess severity of disease we used endoscopic evaluation with the Mayo score which has its issues with interobserver reliability as described in the introduction. All the samples taken forward to RNAseq were taken by 2 endoscopists well versed in the Mayo score which will reduce the variability of the samples. However, we did not always perform histological examination upon the samples as it was not always clinically relevant. After

collecting a number of samples, we re-evaluated our protocol and appreciated that the addition of histological evaluation would have been helpful. We had not planned for histological evaluation in our grant proposal and so there was no money to do this. Furthermore, we had already not had histology on a number of samples we had already performed. We recognise this as a criticism of the data and in future we propose to perform histology on samples adjacent to the study samples with analysis.

7.2.4. Reposited datasets performed by microarray

Differentially expressed transcripts were generated by RNAseq though many of the reposited datasets were performed using microarray. Therefore, in the multiple probes in RNAseq may have been coded by a single probe in microarray which may lead to under or over representation in the enrichment analysis so leading to false results. However, this likely to have an extremely minimal impact on the overall analysis.

7.2.5. Length of time for transport from endoscopy to laboratory

We did have concern over the time taken from the biopsies taken to the time that they were placed in culture as this could have been as much as 3 hours as the samples had to be transported from St Thomas' where the endoscopy was performed to Guy's where the laboratory is located. Whilst we optimised this time as best we could it was a great challenge to reduce this time further due to the location of the lab, lack of endoscopy at Guy's and HTA approved transport usage. It seems likely that there was degradation of the samples with cell death and reduction in the quality of the RNA during this time and the effect that this had on the experiment is unclear. However, as we were comparing treated with IL-23 vs untreated samples the same conditions applied to both experimental groups.

7.2.6. Housekeeper selection for RT-qPCR was not optimised

Selection of the housekeeper for RT-qPCR was based on the laboratory's local protocol, longstanding experience, and preference. However, I have subsequently discovered that RT-qPCR reference gene panels exist and are designed to aid selection of the best housekeeper for that individual tissue. Whilst this is unlikely to have a large effect on the experiment outcome it would be prudent if these experiments were to be performed again to optimise the housekeeping gene first.

7.2.7 Lamina propria mononuclear cells

We used LPMCs derived from cellfoam matrices extensively during our experiments though recognise that as a new technique they have not been investigated as to the composition of cells types included. Whilst there are significant practical advantages in using grids rather than digestion and achieve greater numbers of cells over digestion technique, assumptions are made that LPMCs include more immune cells than with grid digestion. We have not performed cell sorting to establish the abundance of cell types which may vary by patient but also by biopsy. Therefore, differential expression may in part be due to altered composition of cell types in a sample rather than the differential expression of immune cells in an individual.

7.2.8 No healthy controls sent for RNAseq

Given our finite financial resources we chose to concentrate on performing RNAseq on patients with ulcerative colitis rather than direct comparison with healthy controls. We appreciate that this may limit the interpretation of the results as the effect of intervention on samples with ulcerative colitis may differ from the effect seen in healthy controls and so confidence in the results is

lessened. However, prudent use of limited funds was required so we chose to focus on the diseased samples.

7.2.9 Ingenuity Pathway Analysis

IPA has multiple limitations and therefore interpretation of the data can be skewed. The method for gathering and interpreting studies is unclear but it curates many thousands of studies but the scrutiny of quality or methods is not performed and so the results must be viewed with some caution as the methodology may be imperfect. Furthermore, the context in which the experiments are performed is unclear and are likely not to be the same disease process or tissue. For example, IL-23 differential expression that we performed may be compared to IL-23 differential expression of lung tissue and therefore with a different cell mix and context may offer insights which are not truly present in colonic tissue. Whilst imperfect IPA permits interpretation of differentially expressed genes which is lacking in other platforms or techniques.

7.2.10 Experimental design

Our experimental design was to stimulate colonic biopsy derived cells with disease relevant cytokines. We were hampered during the design of this study as this approach had not been performed previously and therefore we had to

design the study based on expert, consensus opinion. For example, the concentration of IL-23 and the length of stimulation. Ideally these conditions would have been tested with a series of experiments using differing doses of IL-23 and length of stimulation to obtain the optimum experimental design balancing quality of the RNA (which will degrade the longer the sample is stimulated) versus the fold change of the signal produced. However, given the time and financial restrictions that we had with this study this was unfortunately unrealistic and so 'expert opinion' was the most appropriate option for us. This therefore raises the valid question of whether the transcriptomic effect seen may have significantly differed, in terms of scripts with significant differential expression as well as fold change, which may have enhanced analysis.

The decision to use cellfoam grids rather than use the traditional digestion method to extract LPMCs was based on experiments performed by colleagues in the Hayday group at King's College. They demonstrated that the numbers of cells derived from the colonic biopsies were numerically more and the protocol was simplified with less steps, shorter and was less dependent upon the individual in the laboratory. However, as this was a new technology its use in colonic biopsies has not been well studied and the composition of the cells extracted have not been analysed to review their composition. Therefore, there is a potential for artefact to be introduced into the results as the grid cells may

not be fully representative of the immune cells of the colonic biopsies. It would be prudent to explore this technique further with analysis of the colonic biopsies and grid derived LPMCs to identify the abundance and composition of the cell types. This may be best performed using flow cytometry and/ or single cell sequencing.

7.2.11 RNA sequencing analysis

Principle Component Analysis revealed close alignment by patient rather than stimulated cytokine demonstrating that the patient determines the vast majority of the transcriptomic profile. However, there was an opportunity to improve the analysis by adjusting for donor of origin effects which may have enhanced understanding of the biological effect. Future PCA will incorporate adjustments for donor of origin effects.

7.2.12 Sample collection and RNA extraction not performed on the same date

It is recognised that the samples sent for RNAseq were gathered over a period of 8 months and then had their RNA processed at different time points. This was due to issues with identifying suitable patients, volume of samples that can have RNA extracted reliably on a single day and experimental failure. The samples were exposed to IL-23 on the day of

collection and then placed and stored in qiazol, which maintains the stability of RNA, until sufficient samples were available for RNA extraction. If the experiment were to be repeated, further efforts and laboratory time would be secured to process the samples in batches over a closer time frame to reduce variance.

7.3. Future work

7.3.1. Effect of IL-23 on CD and healthy control using LPMC experiment

The next step will be to take this experimental technique and apply it to CD. Anti-IL12p40 inhibitors have already been shown to be beneficial in CD (Feagan et al. 2016) and anti-IL23p19 inhibitors are in phase 3 trials currently. It would also be interesting to see if the same ability to separate responders and non-responders would be possible using UNITI data.

Furthermore, performing the same experiment in healthy controls would also be of interest as the direct comparison of IL-23 induced transcriptomic change in healthy v UC may highlight the most important genes which are regulated in disease and hence the ones which may be most important to predict response to endpoints.

7.3.2. Analysis of transcriptomic data from anti-IL23p19 trials

Currently there are multiple phase 3 trials for both UC and Crohn's using anti-IL23p19 drugs. As these drugs selectively blockade the IL23R the IL23 LPMC signature that we developed may perform better at predicting response to clinical and endoscopic endpoints. These trials have yet to complete but are expected to report in 2021/2022 with biomarker analysis following that.

7.3.3. Prospective validation cohorts of anti-TNF and anti-IL12p40 patients

No study has ever had a limitation of their n being too large and this study is no different. Prospectively collected, blinded, centrally read endoscopic and histological analysis coupled with a large number of UC patients from around the globe with a sufficient mix of phenotypes of disease and drugs would be an ideal situation. However, this is unlikely to happen. RNAseq is still too expensive and technologically challenging to be performed in clinical practice and so we must rely on RNAseq data produced in clinical trials. As anti-TNFs and anti-IL12p40 drugs are already licensed there is little prospect of any drug company performing any trials soon. Therefore, we have to use the data which is already in the public domain or to which we have been granted access.

7.3.4. Assess the effect of dose response upon stimulation of LPMCs and explants

We chose a concentration of 10ng/ml for the dose of IL-23 to stimulate the LPMCs and explants with which was based on manufacturer's recommendation and prior work performed in the laboratory. However, there may be a more optimal concentration and we could perform further experiments potentially with RT-qPCR rather than RNAseq, due to the time and expense, to ascertain if this may increase the expression of key genes.

7.3.5. Deconvolution and single cell sequencing

Gene expression of whole tissue will comprise of multiple cell types all producing their unique transcriptional profile. As the tissue will contain an abundance of some cell types and a minority of another there is likely to be an undervaluation or clouding of the smaller number of cell's transcriptional profile. Furthermore, if comparing samples from different patients or indeed various parts of the colon then the composition of cell types may vary and so the transcriptional profile is unlikely to be similar due to differing composition of cell types rather than different biologic process.

Single cell sequencing analyses the transcriptional profile of individual cells within the tissue and therefore an accurate picture can be created of the abundance and effect of the individual cell types. Since the commencement of this project single cell sequencing has been used in CD to demonstrate an inflammatory module associated with anti-TNF non response (Martin et al. 2018). However, single cell sequencing is awfully expensive, requires significant scientific infrastructure and bioinformatic expertise.

Deconvolution is a computed model which using baseline transcriptomic features of each cell type and can be used to estimate the proportion of cell types within a particular sample (Cobos et al. 2020). Deconvolution may have been informative of the change in cell types when stimulated by IL-23 in our LPMC experiment and may have been helpful to identify the relevant cells which are enacting the majority of the transcriptomic change that was seen.

7.3.6. Comparison of different stimulation times

Further to the discussion point regarding length of time of stimulation with IL-23, to evaluate the optimal time of stimulation such that we generated the most significant regulation of individual genes we could repeat the experiment with differing lengths of stimulation to determine the optimal. This could be performed using RT-qPCR of genes downstream of IL-23 (i.e., Th17 pathway

and IFNγ) to determine the optimal amount of stimulation required to maximally regulate these genes.

7.3.7. Alternative cell culture technique

A major issue with both the explant and LPMC experiments was the poor quality of RNA. Despite treating all the samples with the same care and attention with the same protocol and conditions there were a number of samples that were inappropriate for RNAseq analysis (4/9 of explants and 7/14 of LPMCs). Investigating other methods of cell culture would be sensible to improve the yield.

Arguably the most promising technique is to use epithelial colonoids or 3D mini-guts. In brief epithelial stem cells can be derived from diseased tissue and encouraged to grow and multiply in culture. The advantage is that the genetic architecture of the cells remains and the RNA quality that can be derived is of excellent quality and quantity. Having establish the colonoids they can be exposed to a host of stimuli such as cytokines. This technique has already been used by our group to study the effect of IL-22 with high quality results (Powell et al. 2019)

7.3.8. Machine learning would improve identification of predictive factors

To generate a gene signature from differentially expressed analyses we experimented with a number of gene signatures created by analysing gene signatures using a variety of significance filters (fdr and p <0.05, <0.01, <0.1 etc), direction of regulation (up, down, both) as well as limiting by number (i.e., top 50 or 100). This was a time consuming process, and the gene list was analysed initially for content (i.e., familiar or expected genes) and then some were taken forward for enrichment analysis and AUC analysis.

Undoubtedly this was not an efficient process and I suspect that a gene signature with a better AUC could have been achieved by machine learning.

7.3.9 Identification of treatment naïve patients with active disease

Identification of suitable patients proved to be a major limitation of the project where we struggled to identify appropriate patients and then place them on appropriate endoscopy lists such that we obtain biopsies.

The recently published PROFILE trial has shown that identifying newly diagnosed patients was logistically possible and would have provided great benefits to this study. In my current place of work we have created an endoscopy list specifically for patients who we feel are likely to have IBD based on clinical and biochemical data. Such a list would have provided an enriched pool of newly diagnosed IBD patients which would have been very useful for this and other studies.

7.4. The future of personalised IBD medicine

IBD therapies are undergoing an exciting revolution with new biologic and small molecule therapies targeting cytokines, cytokine signalling and trafficking of leucocytes emerging into clinical practice. However, clinical trial and real life

data show that the response to these therapies is heterogeneous and clinical, demographic, and biochemical data have limited utility in predicting response to therapy. Interrogating host immunity has revealed a number of potential biomarkers with the potential for exploitation as predictive precision medicine tools. Transcriptomics is undergoing a paradigm change, partially due to dropping costs of technologies, but also increasing expertise and access to bioinformatics. Promising studies have demonstrated differential expression of keys genes that can be harnessed as predictive biomarkers to discriminate responders and non-responders to IBD therapies. It is foreseeable that a dedicated colonic biopsy for RNA analysis is taken at the time of colonoscopy, as it could provide in-depth insights into immune biology and inform clinical decisions especially if combined with analysis of pre-selected gene signatures such as our IL23 LPMC signature.

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