

**Chronic inflammation and its effects in
Inflammatory Bowel Disease – study of
deep histology, tissue remodelling and
biomarkers, with clinical outcomes**

By

Dr. Uday Nagesh Shivaji

MBBS, PGDip, FRCPS (Glasgow) FRCP(London)

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Medical and Dental School
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Abstract

Inflammatory Bowel Diseases (IBD), mainly comprising of Ulcerative Colitis (UC) and Crohn's Disease (CD) are chronic inflammatory conditions characterised by phases of remission and relapse, sometimes despite treatment. They can cause significant physical and psychological morbidity, and unfortunately increasing in both incidence and prevalence globally. With better understanding of pathogenesis, better description of immunological pathways, treatment options have increased, in particular immunological therapies that have revolutionised management of IBD. However, patients lose response to most therapies over time and finding newer targets remains a subject of ongoing research.

There are several complications that occur in IBD, and most are considered outcomes of chronic inflammation (CI) in the gut. Uncontrolled CI could be due to late diagnosis, suboptimal therapy, loss of response to therapies, or because inflammation at the tissue level remains obscure and unrecognised. By undertaking research with strong, pragmatic methodology, using advanced technology with a multi-omics approach, and conducting deep immunological analysis of intestinal tissue, I have been able to demonstrate that sustained CI can remain active at the tissue level and indeed has detrimental effects at all stages of disease.

I have conducted a series of retrospective studies in different cohorts of IBD to investigate clinical outcomes of sustained CI. In these studies, I have reported that deep histological analysis shows CI and muscular hypertrophy to be dominant features within CD-related fibrotic strictures; delayed IBD diagnosis in inception patients leads to worse clinical outcomes up to 4 consecutive years; elective and non-elective discontinuation of biological therapy in IBD results in poor clinical outcomes. Using these results, I also designed a prospective study (TRAFIC Study) to investigate molecular signals and biomarkers of CI in IBD.

Applying multiomics to analyse intestinal tissue (post-surgical resection of CD-related strictures), and using immunohistochemistry (IHC) techniques, I have demonstrated that gene expression within the stricture is different compared to non-strictures (margins).

The specific interrogation of the JAK-STAT pathway showed increased gene expression of JAK1, STAT1, STAT2 & STAT3 within the stricture, compared to margins. This suggests that JAK1 inhibitors could have a role as anti-inflammatory therapies for stricturing disease.

IHC of selected novel cytokines Interleukin-33 and Interleukin-36gamma, demonstrated high expression levels within the strictures and margins. By correlating these findings with clinical outcomes, our results suggest that IL-36g could be a potential target of therapy for CD and may also have antifibrotic potential. The therapeutic potential of IL-33 is less clear mainly because of significant pleiotropic effects and cannot be recommended at this time.

Combined detection of IL-33 and IL-36g in resection margins could assist in stratifying patients at risk of early post-operative recurrence, making them potential biomarkers in IBD.

These pathways and cytokines of interest are promising targets of therapy, but further confirmation is essential before harnessing their potential, which is currently underway.

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Dedication

My thesis is dedicated to my dear parents who have made endless sacrifices for my success, my wife who has been my solid pillar of support, friend, advisor, companion, confidante at all times, and my son whose love is an inspiration making it all worthwhile. Without their patience and understanding, this work would not have been possible.

Special dedication and tribute

I would like to dedicate this work and pay my respects to my late friend Dr. Louisa Jeffery. We lost Dr. Jeffery very suddenly at a young age and her sad untimely demise came as a shock. Dr. Jeffery was part of our core research team. She was a kind, dedicated, accomplished and brilliant post-doctoral scientist at the University. She was not only my lab co-supervisor but also a very dear friend. She supported me through large part of my work and was integral in the setup of many studies and collaborations throughout our time. I feel a deep sense of loss and her absence has left a huge void. I pray that her soul Rests in Peace and pray for strength for her family.

A special dedication would be one small way of paying my respects to her.

Collaborations achieved

The process of building research collaborations during this study be it local, regional or international, has taught me immensely. At a local level, during study set-up I have had multiple discussions with several stakeholders within the hospital and University, such as the Colorectal surgeons, Pathologists, Radiologists, Gastroenterologists and lab team members to ensure smooth flow of patient recruitment and collection of biomaterials critical for the success of the study.

With guidance from my supervisors, I have had discussions and negotiations with regional teams in the West Midlands area to include them as participating sites in the study, which gave me useful insights into regulatory processes involved and improved my negotiating skills. I was successful in capturing their interest and enrolling them as participating sites. However, this process had to be paused due to the COVID-19 crisis.

I have also been successful at building international collaborations with multiple teams in India. I was involved in discussing with two centres in Bangalore and Kolkata in India and enrolling them as sites for the TRAFIC Study. With help from my supervisors, we were able to build intellectual collaborations and these centres have now recruited a respectable number of patients in the TRAFIC Study. This challenging but fulfilling process has given me a sense of achievement. I have also been able to build stand-alone collaborations with reputed researchers at the Indian Institute

of Science (IISc, Bangalore, INDIA) which is one of the most reputed premier institutes in the country. This has been a fruitful partnership for both teams.

The process of discussions, negotiating and aligning research interests all taught me immensely over the last couple of years. Overall, it has not only been an excellent learning process but also a very rewarding and fulfilling experience.

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The work accomplished here would not have been possible without the support, expertise, encouragement, and dedication of a large number of colleagues and friends involved. In particular, I would like to express my illimitable gratitude to my supervisors and mentors Professor Subrata Ghosh and Professor Marietta Iacucci, without whose vision and support this work would have been impossible. I especially thank them for giving me the opportunity to be part of the excellent team, providing guidance, continuous support, and the freedom to implement ideas throughout my time. Prof Ghosh has been very supportive throughout my study period and beyond. He has given me the perfect balance of advice, independence of thought & action, support, and guidance to complete my work to a high standard. Professor Iacucci has been a mentor and a pillar of support during the entire time I have worked at the University. She has been especially helpful and kind during uncertain times of the pandemic. She has ensured that the work has progressed despite multiple challenges both during and the post-pandemic recovery period. I am grateful for her trust and generosity in giving me the opportunity to take over some prestigious cutting edge academic projects in the field of gastroenterology and endoscopy.

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It would not have been possible to deliver the lab work without the support of our lab team. I would especially like to express my gratitude to Dr. Louisa Jeffery, our post-doctoral fellow who supervised and supported the lab components of my thesis. The other team members Dr. Evaggelia Liaskou, Dr. Stuart Hunter and Ms. Sarah Akiror have been key in delivering our studies. I would like to give a special mention for Dr. Gary Reynolds who is an expert at molecular work, histology and immunohistochemistry and has been extremely helpful in guiding me through some of the challenging aspects of staining the tissue specimens. This work was critical to the theme of this work. I am very grateful for his guidance, patience, and support during my time in the labs. I am grateful to our Pathologists from University Hospitals Birmingham Dr Kassiani Skordilis, Dr. Davide Zardo, Dr. Zainab Abdawn and Dr. Rahul Hejmadi who have been very dedicated to the studies and supported us throughout despite their busy schedules in the hospital. I am also immensely grateful for the support extended by various clinical teams which made it possible to recruit patients and deliver the studies. In particular, Professor Thomas Pinkney has been one of the pillars of the TRAFIC Study. He has been extremely helpful during challenging times, and it would not have been possible to deliver this work without his support. During the uncertain period faced by me due to team restructuring during the pandemic, Prof Pinkney kindly stepped in to guide me through some difficult stages. I would like to especially thank Dr. Kate Kane who was the Clinical Lead of Gastroenterology services during my study period. Dr. Kane's unwavering support and strong guiding spirit helped me through many different challenges during this time. I am indebted to her for being an excellent mentor not only as a clinician but my guide for any difficult circumstances I have faced. Her help has been integral to the successful delivery of my work over the last three years.

I would also like to express my gratitude to my collaborators for their time for intellectual discussions, contribution, and their willingness to work together. Due to their generosity, we have been able to take steps and help translate our scientific ideas into reality. I am grateful to the collaborating team in India led by Dr. Rangarajan Kasturi as the Principal Investigator. This team forms the international recruiting centre for the TRAFIC study. I am also grateful to Dr. Alben Sigamani as the Head of Clinical Research, Dr. Snehali Majumder as the postdoctoral scientist instrumental in setting up the study in India, and Suparna Mukherjee as the lead Specialty Dietician in the study.

Among my collaborators, in particular I would like to thank Professor Sandhya Visweswariah (Indian Institute of Science) for her invaluable inputs during our experiments looking into the guanylate cyclase pathway in IBD. As a pioneering scientist in this field, it was extremely useful to have direction from her. I am also pleased that this initial work with Prof Visweswariah catalysed a further collaboration in India to set-up a study called the SEQUIN-PIBD study along with Dr. Sahana Shankar, looking into Whole Exome Sequencing in Paediatric Inflammatory Bowel Disease.

Last but not the least, I would like to thank the respective Directors of Institutes at the University of Birmingham. I am very grateful to Professor David Wraith who has been extremely kind and understanding during all the challenging circumstances encountered during my study period and beyond. His calm and composed presence in itself were very reassuring, backed by his kindness and support. I would like to thank Professor Philip Newsome for his strong support as the Director of the Biomedical Research Centre (BRC) and Dr. Victoria Day, the Manager of the BRC for her help during times of crisis. I would like to thank Professor David Withers, the Head of Postgraduate Research for his advice, understanding and kindness during times of personal difficulties.

Impact of COVID-19 pandemic and Statement on Disruption to Research

There was significant disruption to my research work due to the pandemic, as explained in detail in this section. My study period for the PhD was from March 2018 to March 2021. The thesis related work was on track until March 2020, after which we went into lockdown. This meant that the lockdown had a severe negative impact during the critical last year of my study period, as was expected.

The research work had to be suspended during the first lockdown as per government guidance, including wet-lab work. A lot of the planned experiments were stopped and unfortunately a number of our consumables went out of date, and we could not recover the losses. I had several discussions with my supervisors, and we decided to stop this work entirely resulting in the loss of an entire chapter.

During the first lockdown I was redeployed from research work towards clinical work (as a contingent worker) for the entire duration of the pandemic. This was done in accordance with the Department of Health guidance at the time. I was working as part of the clinical team between March 2020 and September 2020 and then as part of the frontline workforce until September 2021. During the pandemic, there was also loss of funding due to necessary diversion towards COVID related research. I was therefore required to suspend my work and return to clinical training. During this time, I opted to work less-than-full-time to look after my young family and also planned to complete some of my pending research work in my own time. Despite my best efforts, a lot of the planned work could not be completed due to many reasons- changes in work patterns, lab restrictions, shortage of funds, lack of experts in labs, lack of tissue due to paused

patient recruitment, global disruption in supply chain of consumables, uncertainty, among others. Added to this, the second lockdown also affected my work due to changing team structure. This eventually led to suspension of initially conceived and planned experiments. Two important pieces of work were affected in particular - lab work related to drug repurposing in IBD, and work related to further exploration of Guanylate cyclase pathway as a potential biomarker and target of therapy in IBD. Unfortunately, these projects had to be prematurely closed down entirely, despite the promising initial signals.

Further, there was significant restructuring within the team driven by the pandemic resulting in some attrition of expertise at all levels.

Overall, the disruption during the pandemic resulted in a major change in strategy of my work. Although the bulk of my work for the thesis had already been done, some critical aspects could not be completed. Despite the challenges, with guidance from my supervisors, support from the lab experts and understanding of the Postgraduate office, I was able to finish the work to a high standard with a slightly modified strategy. However, some small sections have remained unfinished, and I request the panel and examiners to kindly consider the above challenges when appraising the submitted work.

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LIST OF ABBREVIATIONS

A

ACCESS - Asia-Pacific Crohn's and Colitis
Epidemiology
AI – Acute inflammation
ALTER - Agitated Low Temperature Epitope
Retrieval
AO - Adverse outcomes
AP -1 - Activator protein 1

B

BRC - Biomedical Research Centre
BSG – British Society of Gastroenterology

C

CARD15 - Caspase activating recruitment
domain 15
CD – Crohn's disease
CDMI - Crohn's Disease MRI Index
cDNA - Complementary DNA
CI – Chronic inflammation
CIS - Cytokine signalling inhibitor
CLGR - Centre for Liver and Gastroenterology
Research
CRC – Colorectal cancer
CRP - C-reactive protein
CT - Computed Tomography
CTE - CT enterography
CTGF - Connective tissue growth factor

D

DALY - Disability-adjusted life-years
DAMPs - Damage-associated molecular
patterns
DNA – Deoxyribonucleic acid
DNase I - Deoxyribonuclease I

E

M

MACS – Magnetic Activated Cell Sorting
MAPK - Mitogen-activated protein kinase
MDT - Multidisciplinary Team
MH - Mucosal healing
MPH - Muscularis propria hyperplasia
MRE - Magnetic resonance enterography
MTA - Material Transfer Agreement

N

NF-κB - Nuclear factor kappa B
NH - neuronal hypertrophy
NHS - National Health Services
NICE - National Institute for Health and Care
Excellence
NK cell - Natural killer cell
NOD2 - Nucleotide Binding Oligomerization
Domain Containing 2

O

OGD – Oesophagogastroduodenoscopy

P

PBMC - peripheral blood mononuclear cell
PDGF - Platelet derived growth factor
PIS - Patient information sheet
PLF - Per low power field
PLS-DA - Partial least squares discriminant
analysis
PMN - Polymorphonuclear cells
PMTS - Possible maximum total score
PTP - Protein tyrosine phosphatase

R

RA - Rheumatoid Arthritis
REC - Research Ethics Committee

ECCO – European Crohn’s and Colitis Organisation

ECM – Extracellular matrix

EMR - Electronic Medical Records

EMT - Epithelial-mesenchymal transition

EndoMT - Endothelial-mesenchymal transition

ESR- Erythrocyte sedimentation rate

ES - Expression score

F

FCP - faecal calprotectin

FS – fibrosis

G

GBD - Global Burden of Disease

GP - General Practitioner

GM-CSF - Granulocyte–macrophage colony-stimulating factor

I

IBD – Inflammatory bowel disease

IFN – Interferon

IHC – Immunohistochemistry

IL – Interleukin

ILC - innate lymphoid cells

IMM-AGE – Immune aging

IPAA - Ileal Pouch-Anal Anastomosis

IRA – Ileorectal anastomosis

IRF - interferon regulatory factor

J

JAK - Janus kinase

K

KEGG - Kyoto Encyclopaedia of Genes and Genomes

K-M – Kaplan – Meier

RIN - RNA integrity number

RNA – Ribonucleic acid

S

SDI - Socio-demographic Index

SIV - Site Initiation Visit

SLE – Systemic lupus erythematosus

SOCS - Suppressor of Cytokine Signalling

SSPS - Statistical Software for Social Sciences

STAT - Signal transducers and activators of transcription

ST2 –Suppressor of tumorigenicity 2

T

Th - T-helper

TGFβ1 – Transforming growth factor β1

TNF alpha - anti-tumour necrosis factor alpha

TPM - Transcripts per million

TRAFIC - Tissue remodelling and fibrostenosis in Crohn’s disease

Treg – Regulatory T cell

TYK2 - Tyrosine Kinase 2

U

UC – Ulcerative colitis

UI – Uncertainty intervals

UoB – University of Birmingham

UMI - Unique molecular index

V

VE - Volume expansion

W

WCE – Wireless capsule endoscopy

Y

YLD - Years lived with disability

YLL - Years of life lost

DISSEMINATION

Publications

Original Research articles relevant to my thesis

Shivaji U N, Evans M, Critchlow T, et al.

Chronic inflammation and other changes are significant components of clinically fibrotic strictures in Crohn's disease: a histological study of resected strictures clinically characterized as noninflamed.

Eur J Gastroenterol Hepatol. 2020 Jul 3. doi: 10.1097/MEG.0000000000001796.

Shivaji U N, Bazarova A, Critchlow T, et al

Clinical outcomes, predictors of prognosis and health economics consequences in IBD patients after discontinuation of the first biological therapy.

Therap Adv Gastroenterol. 2020;13:1756284820981216.

Review articles relevant to thesis

Shivaji UN, Nardone OM, Cannatelli R, Smith SC, Ghosh S, Iacucci M.

Small molecule oral targeted therapies in ulcerative colitis

Lancet Gastroenterol Hepatol. 2020;S2468-1253(19)30414-5. doi:10.1016/S2468-1253(19)30414-5

Shivaji UN, Sharratt C, Thomas T, Smith S, Iacucci M, Moran G, ... Bhala N. (2019)

Review article: managing the adverse events caused by anti-TNF therapy in inflammatory bowel disease.

Alimentary Pharmacology & Therapeutics, (6), doi: [10.1111/apt.15097](https://doi.org/10.1111/apt.15097)

Nardone O, **Shivaji UN**, Ferruzza V, Ghosh S, Iacucci M. (2019)

Soluble Blood Markers of Mucosal Healing in Inflammatory Bowel Disease: The Future of Noninvasive Monitoring.

Inflammatory Bowel Diseases, doi: [10.1093/ibd/izz226](https://doi.org/10.1093/ibd/izz226)

Majumder S, **Shivaji U N**, Kasturi R, Sigamani A, Ghosh S, Iacucci M.

Inflammatory bowel disease-related colorectal cancer: Past, present and future perspectives.

World J Gastrointest Oncol. 2022;14(3):547-67. doi: 10.4251/wjgo.v14.i3.547

Oral presentations at conferences

Abhishek Rao, Alina Bazarova, Pathrabali Mitra, Snehal Majumder, Tommaso Lorenzo Parigi,

Subrata Ghosh, Marietta Iacucci, **Uday N Shivaji**

Delayed IBD diagnosis leads to worse outcomes over years, with multiple implications on service delivery.

Presented at British Society of Gastroenterology Conference June 2022

Doi: Gut 2022;71:A5-A6. DOI- 10.1136/gutjnl-2022-BSG.9

International poster presentations and abstracts

Abhishek Rao, Alina Bazarova, Pathrabali Mitra, Snehali Majumder, Tommaso Lorenzo Parigi, Subrata Ghosh, Marietta Iacucci, **Uday N Shivaji**

Delayed IBD diagnosis increases risk of requiring biologics in first 4-years -an inception cohort study. *Doi: Gut 71(Suppl 1):A56.2-A57. DOI:10.1136/gutjnl-2022-BSG.99*

*Akiror S, Acharjee A, Jeffery L, **Shivaji, UN** et al. Sa132*

Transcriptome analysis identifies dysregulated pathways and targets for therapy in patients undergoing surgical resection for Crohn's disease.

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Shivaji U, Bazarova A, Critchlow T, Smith S, Nardone O, Love M, ... Iacucci M. (2020). P220

Predictors of prognosis after discontinuation of first-use biologics in IBD patients with at least 24 months follow-up. *Journal of Crohn's and Colitis*, (Supplement_1), doi: [10.1093/ecco-jcc/jjz203.349](https://doi.org/10.1093/ecco-jcc/jjz203.349)

Shivaji U, Bazarova A, Critchlow T, Nardone O, Smith S, Love M, ... Iacucci M. (2020). P435

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Regulators of the guanylate cyclase pathway are potential novel markers of mucosal healing in IBD. *Journal of Crohn's and Colitis*, (Supplement_1), doi: [10.1093/ecco-jcc/jjz203.134](https://doi.org/10.1093/ecco-jcc/jjz203.134)

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The anti-inflammatory effects of niclosamide on cytokines produced by PBMCs derived from IBD patients. *Journal of Crohn's and Colitis*, (Supplement_1), doi: [10.1093/ecco-jcc/jjy222.220](https://doi.org/10.1093/ecco-jcc/jjy222.220)

Shivaji U, Evans M, Critchlow T, Smith S, Iacucci M, Ghosh S, ... Skordilis K. (2019). P222

Purely fibrostenotic Crohn's disease without chronic inflammation is uncommon – a histopathologic study of resected intestine. *Journal of Crohn's and Colitis*, (Supplement_1), doi: [10.1093/ecco-jcc/jjy222.346](https://doi.org/10.1093/ecco-jcc/jjy222.346)

Shivaji U, Evans M, Critchlow T, Gui S, Smith S, Iacucci M, ... Skordilis K. (2019). Sa1817 – Fibrosis Constitutes Less Than 50% of the Total Volume in a Resected Fibrostenotic Stricture in Crohn's Disease. *Gastroenterology*, (6), doi: [10.1016/S0016-5085\(19\)37884-9](https://doi.org/10.1016/S0016-5085(19)37884-9)

Nardone O, Jeffery L, Bazarova A, Acharjee A, Gkoutos G, Zardo D, **Shivaji UN**... Iacucci M. (2020). P297 Novel serum soluble markers of histological healing in ulcerative colitis. *Journal of Crohn's and Colitis*, (Supplement_1), doi: [10.1093/ecco-jcc/jjz203.426](https://doi.org/10.1093/ecco-jcc/jjz203.426)

A brief outline of each chapter in the thesis is as follows-

Chapter 1 – This chapter is a general introduction to the topic of Inflammatory Bowel Diseases (IBD). This covers the epidemiological trends of IBD across the globe, the pathogenesis, morbidity due to disease burden across the world and also management of Ulcerative Colitis (UC) and Crohn's Disease (CD). In addition, this covers the topic of chronic inflammation, its related complications in IBD, how this area of research interest shaped our study and gives an overview of the overall project set up.

Chapter 2 – This chapter is a discussion on Phase 1 of our work. A retrospective study of semi-quantitative histological analysis of Crohn's Disease related fibrotic strictures in patients who had undergone surgical resection. This study showed predominant presence of chronic inflammation in strictures, even though these strictures had been clinically and radiologically deemed 'cold' fibrotic strictures.

Chapter 3– This chapter is a discussion on Phase 2 of our work. The overall aims were to study the clinical outcomes of suboptimal control of chronic inflammation in IBD.

The first section of this chapter is a discussion on clinical outcomes of delayed diagnosis of IBD. The aim of the study was to investigate if different types of delay led to poorer outcomes in the long term. This untreated cohort of patients is a surrogate for ongoing chronic inflammation and the effects of the same.

The second section of this chapter is clinical outcomes in IBD patients who have discontinued biological therapy, for multiple reasons. The aim of the study was to examine adverse outcomes

of suboptimal control of inflammation and the resulting economic burden on the health system. The results confirm the importance of controlling inflammation aggressively to minimise complications and disease progression, which reinforces the findings of all our work.

Chapter 4 – This chapter is a discussion on the prospective non-interventional study called TRAFIC (Tissue Remodelling in Fibro-stenosing Crohn’s Disease) Study, which was designed based on the results of our retrospective study. The aim was to carry out molecular work and investigate novel biomarkers of inflammation-fibrosis pathways,

The first section in this chapter discusses details about study set-up process & experience, study design, methodology; the clinical data is presented along with results of RNA sequencing of strictures in CD. The analysis allowed us to examine existing and novel pathways and biomarkers. The second section of this chapter includes detailed report on immunohistochemistry of specific novel cytokines relevant in inflammation and fibrosis that we explored as potential targets of therapy.

1 CHAPTER 1: GENERAL INTRODUCTION AND BACKGROUND TO CHRONIC INFLAMMATION IN INFLAMMATORY BOWEL DISEASE

Parts of this chapter have been published as-

Shivaji UN, Nardone OM, Cannatelli R, Smith SC, Ghosh S, Iacucci M.

Small molecule oral targeted therapies in ulcerative colitis.

Lancet Gastroenterol Hepatol. 2020 Sep;5(9):850-861. doi: 10.1016/S2468-1253(19)30414-5.

Shivaji U N, Sharratt C L, Thomas T, et al.

Review article: managing the adverse events caused by anti-TNF therapy in inflammatory bowel disease.

Aliment Pharmacol Ther. 2019;49(6):664-80. doi: 10.1111/apt.15097

Majumder S, **Shivaji U N**, Kasturi R, Sigamani A, Ghosh S, Iacucci M.

Inflammatory bowel disease-related colorectal cancer: Past, present and future perspectives.

World J Gastrointest Oncol. 2022;14(3):547-67. (Majumder, Shivaji et al. 2022)

Overview of chapter

The introduction chapter gives an overview of multiple aspects of IBD. The first section includes epidemiology, trends of prevalence globally, brief discussion of pathogenesis and a succinct summary of management options for both UC and CD. The next section describes chronic inflammation, the drivers of this, and also the resulting early and late complications in patients. The last section describes how the work plan for our research project evolved and how each stage was planned and implemented.

1.1 Section 1A - Introduction

1.1.1 Background

Inflammatory Bowel Disease (IBD) is a chronic inflammatory condition that mainly comprises of Ulcerative Colitis (UC) and Crohn's Disease (CD). Although the precise aetiology of IBD remains unknown, there have been several factors identified in its pathogenesis. The condition is usually a result of a complex interplay of genetic predisposition, dietary factors, along with a large number of environmental factors that contribute towards a dysregulated immune system, and this remains a subject of ongoing research.(Ananthakrishnan, Bernstein et al. 2018)

History of IBD

IBD was first reported back in the 19th century and has been described in literature periodically over time.(Kaplan 2015) The first description of an inflammatory condition of the colon was given by Wilks and Moxon in 1875 who also coined the term Ulcerative Colitis in medical literature. A case of regional ileitis first described in 1932 gained recognition as the first case of Crohn's Disease in medical literature.(Crohn, Ginzburg et al. 2000) Since then, there have been two distinct forms of IBD recognised i.e UC and CD.

This chapter discusses different aspects of IBD under the following sections –

- epidemiology of IBD and global burden of disease
- rising incidence in low- and middle-income countries
- possible reasons / hypotheses for this increase globally
- brief description of pathogenesis

1.1.2 Epidemiology of IBD

The epidemiology of IBD has been studied over many decades and there has been a significant change in the distribution of the disease globally. **Figure 1-1** demonstrates the historical timelines of changing epidemiological status since its first description in 1875. (Kaplan 2015) A condition that was predominantly seen in the developed world is now increasing in incidence in the developing areas of the world. Although the highest prevalence remains in developed countries, new epidemiological data suggests that it is now truly a worldwide condition. (Loftus 2004, Molodecky, Soon et al. 2012, Torres, Billioud et al. 2012, Hoivik, Moum et al. 2013, Ungaro, Mehandru et al. 2017, Ng, Shi et al. 2018)

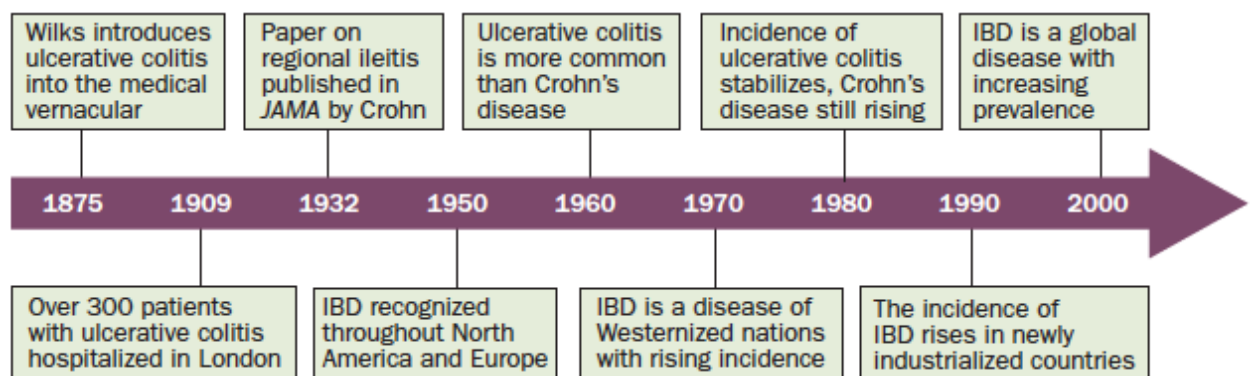


Figure 1-1 Historical timelines of IBD over time

(Figure adapted with permission from Kaplan et al)

A systematic review looking into the incidence and prevalence of IBD spanning decades, included 147 studies reported worldwide between 1990 and 2016. According to this report, the highest prevalence values were in Europe and North America. The prevalence exceeded 0.3% in North America, Oceania, and many countries in Europe. Interestingly, it was noted that incidence of IBD since 1990 has been rising in newly industrialised countries in Africa, Asia, and South America, including Brazil. (Ng, Shi et al. 2018) The authors noted a substantial shift in the epidemiology

wherein incidence of disease after 1990 stabilised in the western world, but prevalence remained high. But in contrast, newly industrialised countries face rising incidence, similar to trends seen in the western world during the latter part of the 20th century.(Ng, Shi et al. 2018)

Kaplan et al mention that these epidemiological differences in IBD between the Western world and newly industrialized countries are primarily explained by genetic and environmental influences on disease development.(Kaplan 2015) It is possible that factors such as recent advances in health-care infrastructure in newly industrialised countries, and methodological challenges in reporting epidemiological data influence differences in reported incidence between countries and world regions.(Kaplan 2015)

As previously mentioned, the highest occurrence of both UC and CD is in the developed nations of Europe and North America.(Burisch, Jess et al. 2013) The highest incidence and prevalence rates within Europe are found in Scandinavia and the United Kingdom, and they remain rare in Eastern Europe.(Burisch, Jess et al. 2013)

The incidence of UC in Europe ranges from 0.9 to 24.3 per 100,000 person-years, and in CD ranges from 0.5 to 10.6 cases per 100,000 person-years. There is a north-south/east-west gradient that has been noted in Europe over decades.(Burisch, Jess et al. 2013) The European Collaborative Study on Inflammatory Bowel Disease (EC-IBD) assessed this north-south gradient in a prospective population-based cohort using uniformed diagnostic criteria and case ascertainment methods. The group reported total incidence rates of 6.3 for CD and 11.4 for UC per 100,000 person-years in northern Europe, while in southern Europe there were lower incidence rates with for 3.6 for CD and 8.0 for UC per 100,000 person-years.(Shivananda, Lennard-Jones et al. 1996)

The incidence and prevalence of IBD in Europe have been robustly and comprehensively summarised by Burisch et al as part of the study conducted by the European Crohn's and Colitis

Organisation Epidemiological Committee (ECCO-EpiCom).(Burisch, Jess et al. 2013) By extrapolating the incidence figures on a total European population (app. 731 million in 2006), the authors reported an indicative maximal estimate of 78,000 new cases of CD and 178,000 new cases of UC each year, and a combined estimate of 256,000 new cases of IBD per year.(Burisch, Jess et al. 2013)

The other geographic area with one of the highest incidence and prevalence rates globally, is North America.(Bernstein, Wajda et al. 2006, Loftus, Loftus et al. 2007) A comprehensive systematic review of population-based studies in IBD was reported by Molodecky et al in 2012.(Molodecky, Soon et al. 2012) According to this study, the annual incidence of UC in North America was reported to be between 0 to 19.2 per 100,000 and between 0 to 20.2 per 100,000 for CD; prevalence was reported to be 37.5 to 248.6 per 100,000 for UC and 16.7 to 318.5 per 100,000 for CD.(Molodecky, Soon et al. 2012)

The incidence and prevalence of IBD have been increasing in other areas of the world. Asia has seen the biggest wave of industrialization in the last 20 years, with regions like Japan, China, India, and Middle East being some of the fastest growing economies of the world. The massive growth and industrialization in these areas have probably contributed to a rapid emergence of IBD in the population.(Ng 2016, Kaplan and Windsor 2021) The Asia-Pacific Crohn's and Colitis Epidemiology (ACCESS) study conducted in 9 countries across Asia-Pacific reported incidence rates ranging from 0.54 to 3.44 per 100,000 population in Asia.(Ng, Tang et al. 2013) The incidence rate (per 100,000 people) for UC was 0.76 and CD was 0.54.

In their study, the highest annual incidence of IBD was seen in highly urbanized areas like Guangzhou (mainland China) at 3.44 per 100,000, followed by Hong Kong at 3.06 per 100,000 and Macau at 2.2 per 100,000 persons. Similar findings have been reported by Wang et al in their

systematic review in 2023 looking into burden of IBD in 204 countries globally. (Wang, Li et al. 2023) As per their findings, by the end of 2019, there were approximately 4.9 million cases of IBD worldwide, with China and the USA having the highest number of cases. A total of 147 out of 204 countries or territories experienced an increase in the age-standardised prevalence rate. It is a worrying trend confirmed by other groups who reported this increasing trend across all age groups including in the elderly age groups. (Caron, Honap et al. 2024)

Overall, IBD now appears to be a global disease with stabilising incidence in western countries, although with a high prevalence of > 0.3%. (Ng, Shi et al. 2018) (Wang, Li et al. 2023) The trend is an increasing incidence in newly industrialised countries whose societies have become more westernised and seen across all groups. (Ng, Shi et al. 2018, Caron, Honap et al. 2024)

Global disease burden due to IBD

The chronic inflammatory nature of IBD adds to the cumulative global disease burden due to high prevalence rates. The global burden of IBD has significantly increased over the last few decades. A study group looking into the Global Burden of Disease (GBD) reported their findings of prevalence, mortality, and overall burden of IBD in 195 countries and territories between 1990 and 2017, based on data from the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2017. (Alatab, Sepanlou et al. 2020) This comprehensive study used indicators such as mortality, prevalence, years of life lost (YLLs) due to premature death, years lived with disability (YLDs), and disability-adjusted life-years (DALYs), to report their findings. (Alatab, Sepanlou et al. 2020) *[In this study, all estimates were reported as numbers and rates per 100 000 population, with 95% uncertainty intervals (UI)].* This is illustrated in **Figure 1-2**.

As per this study, the estimated number of total IBD cases were 6.8 million (95% UI 6.4–7.3) globally in 2017. The age-standardised prevalence rate had increased from 79.5 (75.9–83.5) per 100,000 population in 1990 to 84.3 (79.2–89.9) per 100,000 population in 2017. The highest age-standardised prevalence rate was in high-income North America (422.0 [398.7–446.1] per 100 000) and the lowest rates were observed in the Caribbean (6.7 [6.3–7.2] per 100 000 population). As previously known and expected, high Socio-demographic Index (SDI) locations had the highest age-standardised prevalence rate, while low SDI regions had the lowest age-standardised prevalence rate. **(Figure 1-3)** The investigators also noted that the total YLDs attributed to IBD almost doubled over the study period, from 0.56 million (0.39–0.77) in 1990 to 1.02 million (0.71–1.38) in 2017. The age-standardised rate of DALYs decreased from 26.5 (21.0–33.0) per 100 000 population in 1990 to 23.2 (19.1–27.8) per 100 000 population in 2017.

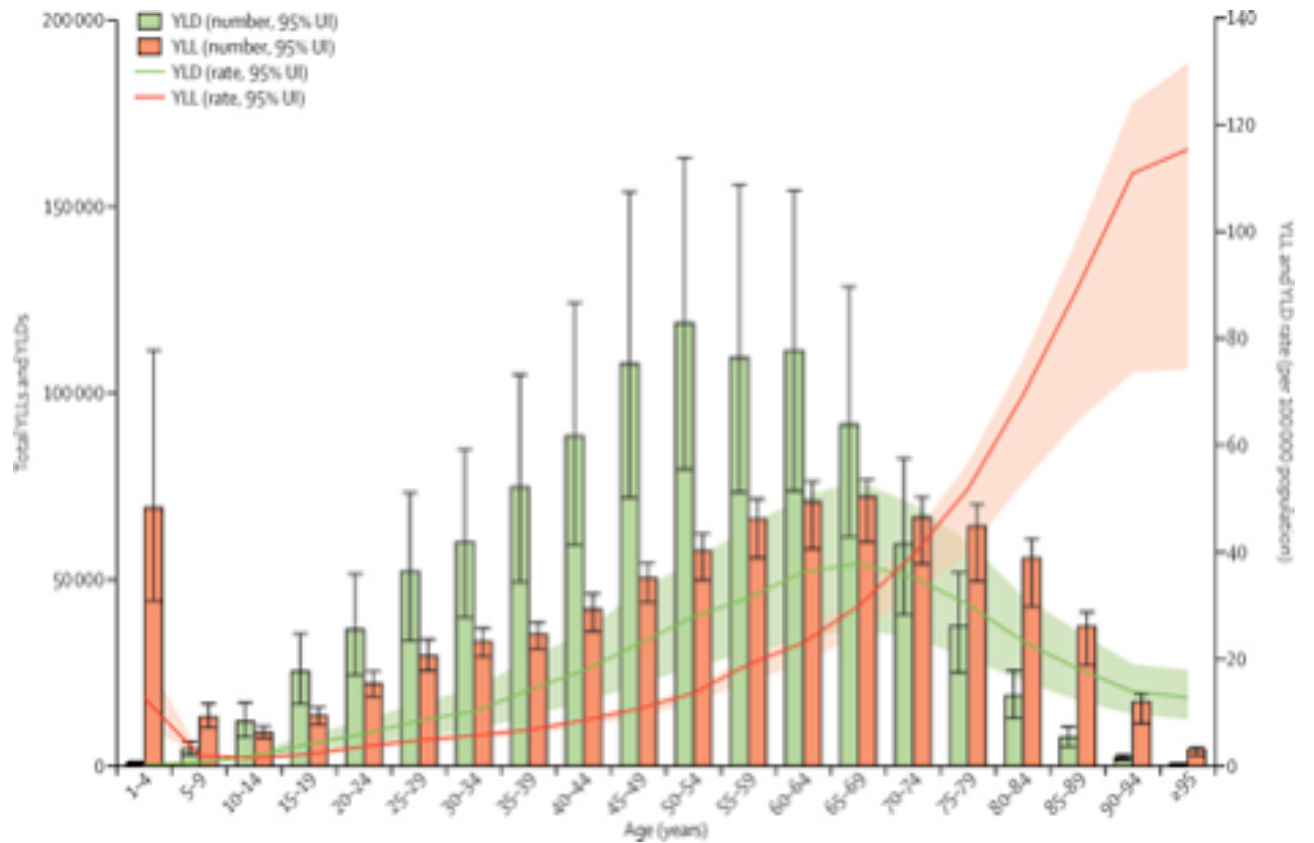


Figure 1-2 Global counts and age-specific rates of YLLs and YLDs due to IBD across age groups, 2017

(Figure adapted with permission from Alatab et al)

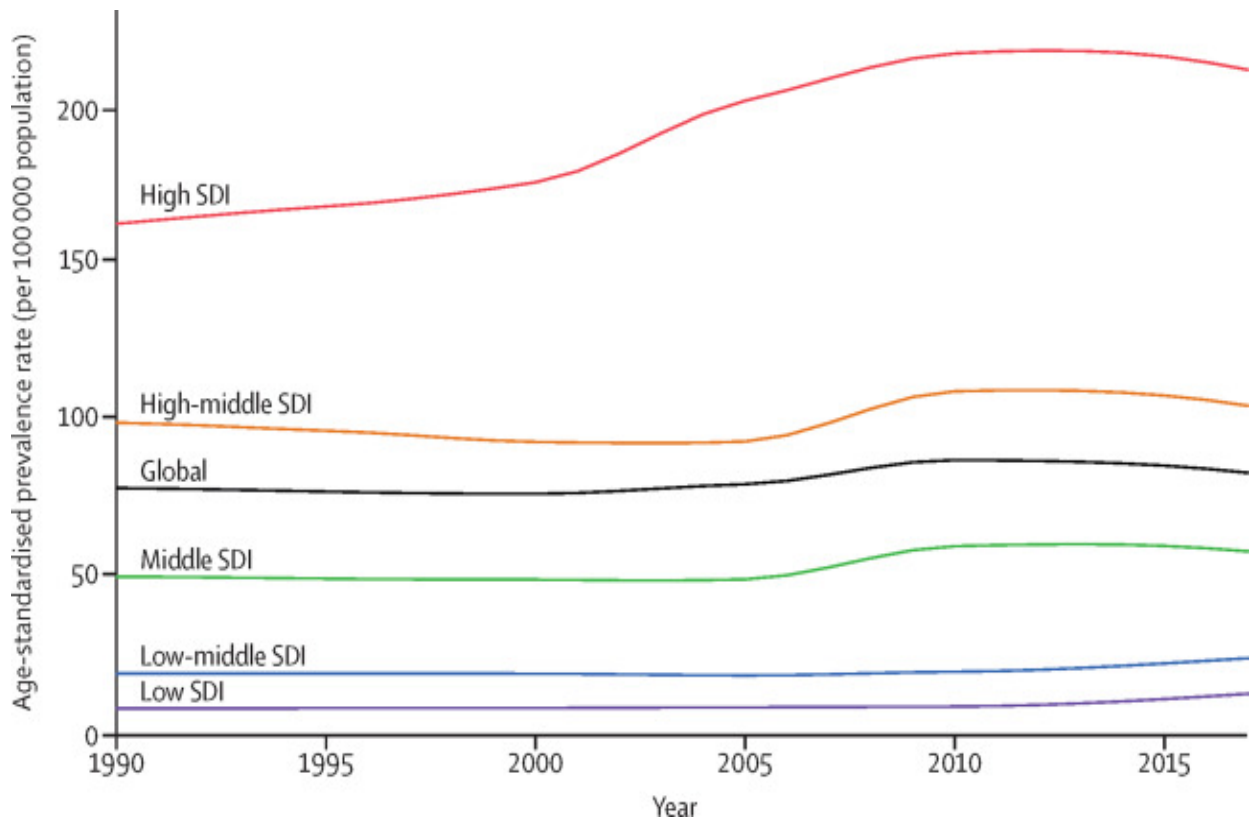


Figure 1-3 Trends from 1990 to 2017 in age-standardised prevalence rates of IBD by SDI quintile

(Figure adapted with permission from Alatab et al)

Rising incidence of IBD in newly industrialised countries with low to middle SDI

The increasing incidence of IBD in regions which were previously thought to be low incidence and prevalence areas, is a concern. In the GBD study, the authors reported an increase in age-standardised prevalence rate of IBD in many such regions including east and south Asia, Oceania, and sub-Saharan Africa (low, low-middle and middle-SDI in **Figure 1-3**). A combination of factors such as improvements in socio-economic status in newly industrialised countries, changes in diet and lifestyle, improved sanitation, changed microbiota and environmental factors increase the risk of developing IBD.(Alatab, Sepanlou et al. 2020) It is also possible that other additional factors within the health care systems of these regions such as increased awareness among physicians and, improved access to health-care systems, more widely available diagnostic tools, might also

contribute to higher rates of diagnosis.(Alatab, Sepanlou et al. 2020) This is a particular issue in South Asia which has two of the most populous countries in the world, China and India. One prospective population-based study in 13 countries in Asia-Pacific between 2011 to 2013 found that India (9.31; 95% CI: 8.38-10.31) and China (3.64; 95% CI, 2.97-4.42) have the highest IBD incidence (per 100,000 persons) in Asia.(Ng, Kaplan et al. 2019) Although the incidence and prevalence figures in these regions remain lower compared to regions such as North America or Europe, it has been highlighted that the number of patients with IBD in newly industrialized countries could approximate that in the Western world as early as 2025, owing to rising prevalence and rapidly growing populations.(Kaplan 2015) It is also true that the peak incidence of IBD has probably not yet transpired in these countries.(Ng, Shi et al. 2018) More recent studies on global burden of disease have reported similar findings confirming the predictions of continued increase in incidences across the globe. (Wang, Li et al. 2023)

This rapidly changing global burden of IBD over the next decade is a major cause for concern. The significant burden placed on the health systems across the world will need to be considered. Managing this chronic condition will require a multi-pronged approach that involves patient education and awareness programmes in the community to prevent delays in diagnosis, high quality research into interventions that could potentially prevent IBD and also find new targets of therapy and improving the current delivery of care to existing patients with IBD.

1.1.3 Pathogenesis of IBD

There has been extensive research into the pathogenesis of IBD over the last few decades. It involves a complex interplay of genetic, environmental, and immunological factors. While the exact cause of IBD remains unknown, it has been hypothesised that a combination of these factors leads to an abnormal immune response against the intestinal microbiota in genetically susceptible individuals. This dysregulated immune response triggers chronic inflammation, resulting in the characteristic symptoms and complications seen in IBD. A brief discussion of each of the factors is given in this section.

Genetic Predisposition: Genetics play a significant role in the development of IBD.(Hanauer 2006)

It is known that UC and CD may be heterogeneous polygenic disorders. They probably share some but not all susceptibility loci.(Satsangi, Morecroft et al. 2003) Monogenic IBD is less common and perhaps more significant in the paediatric population where several genetic mutations have been reported as a cause of early-onset and very-early onset IBD.(Uhlig, Schwerd et al. 2014) Family and twin studies have shown a higher concordance rate among relatives, indicating a hereditary component. Multiple genetic variants have been identified, particularly in genes involved in the immune system and barrier function of the gut. Several genes have been implicated in IBD, including NOD2, ATG16L1, and IL23R, among others. These genes are involved in various aspects of the immune response, such as recognizing and responding to bacterial components, regulating autophagy, and modulating inflammatory pathways.(Aldhous, Nimmo et al. 2003) Genetic variations in these genes can potentially disrupt the balance of immune regulation, making individuals more susceptible to IBD.(Hanauer 2006) It is important to mention that the presence of a mutated gene does not in itself mean development of disease, nor does it predict who will

be affected, highlighting the importance of other factors that are involved in triggering the disease. These genetic factors do influence the susceptibility, severity, and phenotypic characteristics of IBD.(Uhlig, Schwerd et al. 2014) (Hanauer 2006)

Environmental Factors: Environmental factors are believed to play a substantial role in the pathogenesis of IBD. These triggers include several factors such as diet, microbial exposures, antimicrobial therapy, smoking, stress, type of occupations and geographical location. Westernized diets, high in refined sugars and low in fibre, have been associated with an increased risk of IBD.(Hanauer 2006) Dietary studies have so far remained inconclusive and the role of diet in the pathogenesis of IBD is a subject of ongoing research. Exposure to infectious agents, such as certain strains of bacteria or viruses, may trigger an abnormal immune response in genetically susceptible individuals.(Hanauer 2006) Tobacco smoking is the strongest environmental risk factor for IBD. The relationship between IBD and smoking is complex, suggesting unique pathophysiological factors for both UC and CD. Interestingly, smoking has a protective effect in UC, and this has been shown in multiple case-control studies.(Benoni and Nilsson 1987, Lindberg, Tysk et al. 1988) Active smokers carry a lower relative risk of UC compared to non-smokers. Also, the risk of developing UC is higher in ex-smokers compared to non-smokers, and they appear to have a more severe disease course overall (Boyko, Perera et al. 1988), the reasons for which are unclear. Conversely, cigarette smoking is a significant risk factor for CD (Relative Risk(RR), 1.15-3.9).(Lindberg, Tysk et al. 1988) Another study found the RR to be 3.5 (95% CI 1.8–6.6)(Somerville, Logan et al. 1984) Smokers with CD were noted to have a poorer disease course than non-smokers, with higher disease recurrence, more frequent surgical interventions, and a greater need for immunosuppressive agents.(Parkes, Whelan et al. 2014, Kuenzig, Lee et al. 2016)

Host Immunological Dysregulation: The immune system in individuals with IBD demonstrates dysregulated responses, leading to chronic inflammation. The intestinal immune system normally maintains a delicate balance between tolerance to innocuous constituents of the gut and protection against pathogens. There are trillions of microbiota residing within the intestinal lumen. There is a potential of antigens in the microflora, that could have a pro- or anti-inflammatory effects. However, these antigens are present in the body from birth and have a degree of access to the internal environment. As a result of this, they might be subject to the intra-thymic processing that allows the immune system to distinguish self from non-self antigens, and, if so, could be considered to be equivalent to self-antigens.(Bouma and Strober 2003) If there is a reaction mounted to these self-antigens, it could be argued that the host is mounting an autoimmune response.(Bouma and Strober 2003) This immune dysregulation results in chronic mucosal inflammation seen in IBD.

Once triggered, the immune cells, particularly T cells and various subsets of immune cells, become activated and release pro-inflammatory molecules such as cytokines (e.g., tumour necrosis factor- α , interleukins 1,6,13 and 23), chemokines, and reactive oxygen species. These mediators attract more immune cells to the intestinal mucosa, perpetuating the inflammatory process and causing tissue damage. This sustained immune activation leads to chronic inflammation and related complications. Additionally, Treg responses are weaker leading to an imbalance between pro-inflammatory and regulatory-cell responses.(Bouma and Strober 2003)

Intestinal Barrier Dysfunction: In normal health, the healthy intestinal epithelium, which has highly evolved tight junctions provides an effective barrier against luminal microbiota and

antigens. The intestinal epithelial cells have also developed regulatory mechanisms that check inappropriate activation of immune responses.(Podolsky 2002) This integrity of the intestinal barrier, that separates the intestinal lumen from the underlying tissues, is crucial in preventing the entry of harmful substances into the gut wall. If due to any reason bacterial products are able to breach the mucosal barrier, they will come into direct contact with immune cells, resulting in a classic adaptive immune response.(Podolsky 2002) The increased permeability of the epithelial lining of the results in continuous stimulation of the mucosal immune system. In patients with IBD, it has been suggested that this may be the primary defect. (Bouma and Strober 2003, Hanauer 2006) This has been demonstrated in animal studies. Further, the luminal bacteria are known to intensify the permeability defect further, thus establishing a self-sustaining cycle of chronic mucosal inflammation.(Hanauer 2006)

Interactions with Gut Microbiota: The gut microbiota, composed of trillions of microorganisms residing in the intestinal tract, plays a vital role in maintaining gut homeostasis. There has been a significant amount of research and enhanced understanding of the gut microbiota and its role in IBD in recent decades. The intestinal microbiota profoundly affects host immune composition and is likely one of the most important environmental factors in IBD.(Kaser, Zeissig et al. 2010) In individuals with IBD, there is an alteration in the composition and function of the gut microbiota, termed “dysbiosis”. With this initial signal, there have been studies conducted to determine whether specific alterations can be identified in the intestinal microbiota in IBD. 16S rRNA sequencing revealed a detectable difference between the intestinal microbiota in CD and UC compared to healthy controls.(Frank, St Amand et al. 2007) This difference in microbial phylotypes largely arises from a distinct subset of CD and UC patients,(Kaser, Zeissig et al. 2010)

that is characterized by depletion of commensal bacteria with a tenfold lower bacterial load affecting both major classes of commensal phyla, Firmicutes and Bacteroidetes. (Frank, St Amand et al. 2007) Whether this “dysbiosis” in the IBD subset is associated with particular genotypes and therefore a primary effect that is a precursor to inflammation, or whether it is a consequence of inflammation is unknown. (Kaser, Zeissig et al. 2010)

Alterations in the composition and function of the gut microbiota, termed dysbiosis, have been observed in individuals with IBD. Dysbiosis may result from a combination of genetic susceptibility, environmental factors, and immune dysregulation. Changes in the gut microbiota can lead to an imbalanced immune response and perpetuate chronic inflammation in IBD. **Figure 1-4** demonstrates pathogenesis of IBD as described in this section. (Yeshi, Ruscher et al. 2020)

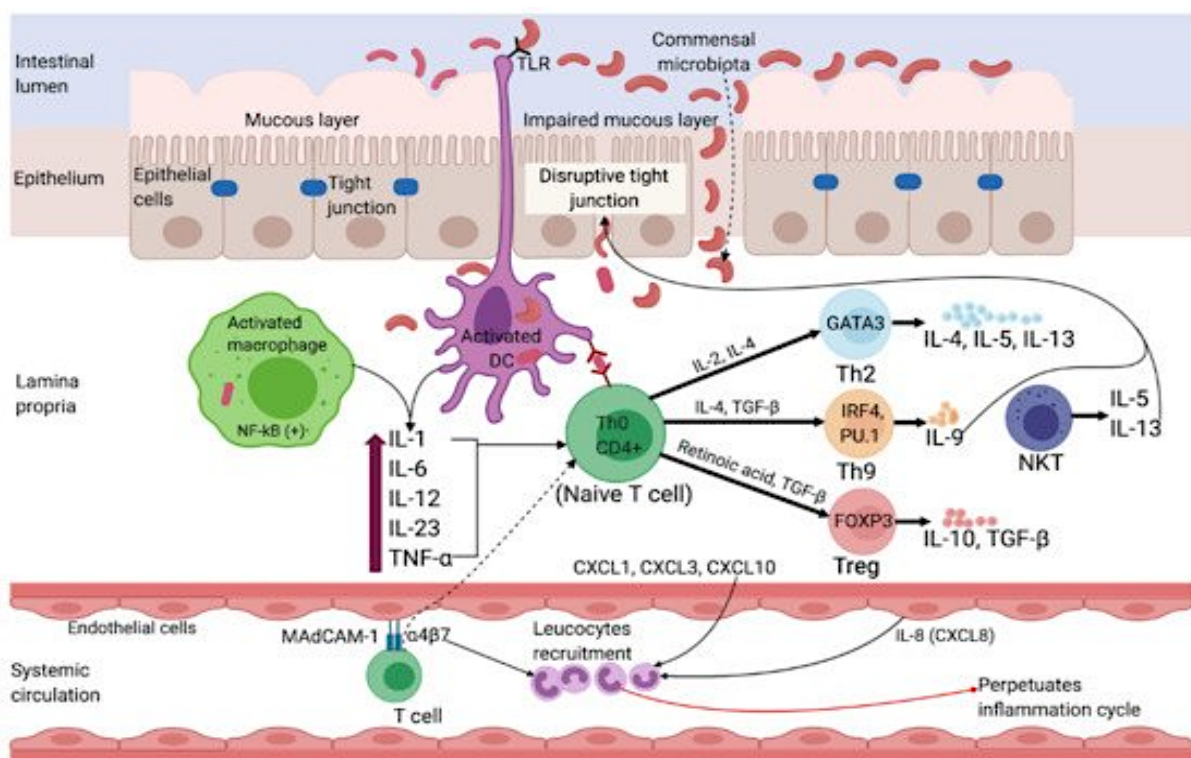


Figure 1-4 Pathogenesis of IBD

(Figure adapted with permission from Yeshi et al)

1.1.4 Diagnostic modalities in IBD

The current available modalities in clinical practice for comprehensive assessment and reliable diagnosis of IBD are briefly discussed here. This begins with a meticulous clinical assessment by the Gastroenterologist and later includes a combination of tests, both non-invasive and invasive. This approach is largely similar globally with minor variations depending on the infrastructure and expertise available in hospitals. There is no single test that helps reach the diagnosis of IBD. It is based on a combination of clinical, biochemical, stool, endoscopic, cross-sectional imaging, and histological investigations.(Maaser, Sturm et al. 2019)

1.1.4.1 Laboratory Testing

Laboratory investigations, including full blood counts, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and faecal calprotectin (FCP), play a crucial role in screening and later in assessing disease activity to guide treatment decisions. Although sensitive and reliable, these markers lack specificity and can be elevated in other conditions. (Maaser, Sturm et al. 2019)

1.1.4.2 Endoscopy

Endoscopic assessment is the most commonly used technique to confirm the diagnosis of IBD. Although invasive, it is reliable and gives a great deal of information useful to guide treatment. *Colonoscopy*: this helps direct visualization of the colon and terminal ileum allowing for mucosal assessment, biopsy collection, and disease classification (discussed earlier). It is an extremely useful test that can help distinguish between CD and UC, evaluate disease extent, assess severity

by macroscopic evaluation of the mucosa (**Figure 1-5**) and detect complications of inflammation such as polyps and cancer.

Upper GI Endoscopy or Oesophagogastroduodenoscopy (OGD): is useful in cases of suspected upper gastrointestinal involvement in CD. (Maaser, Sturm et al. 2019)

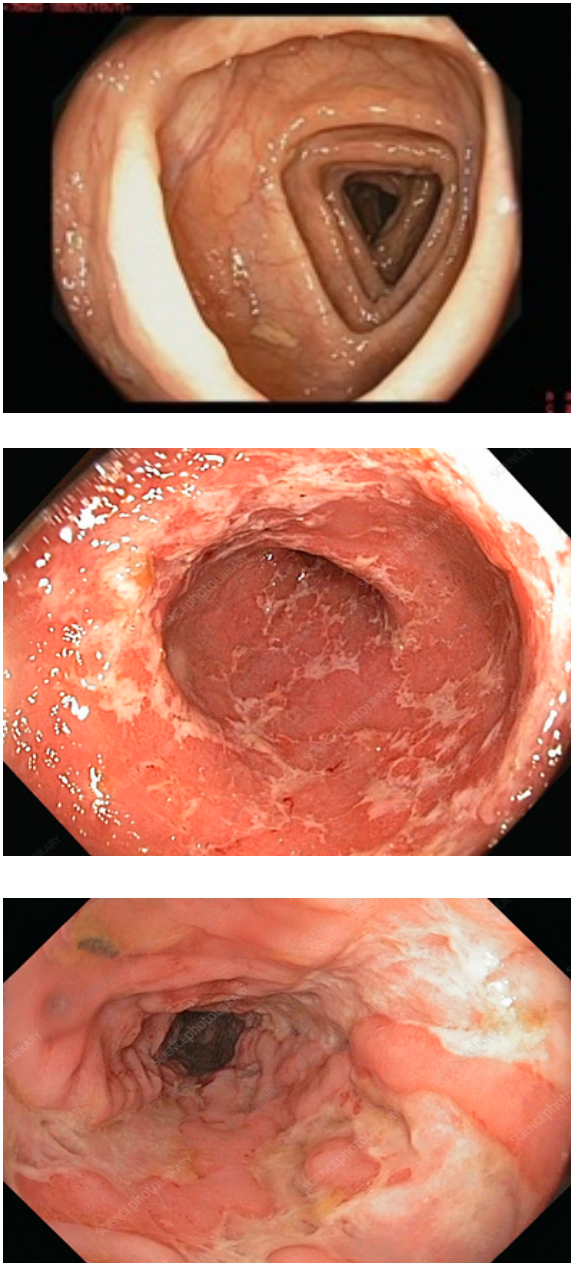


Figure 1-5 Endoscopic images of normal colon, Ulcerative Colitis and Crohn's Disease respectively (above downwards) *(Images credit- Science Photo library with permission)*

1.1.4.3 Imaging Modalities

Radiography: Conventional X-rays continue to be used to look for bowel dilatation, strictures, and perforations. However, their sensitivity is limited, and they are often surpassed by more advanced cross-sectional imaging techniques.

Computed Tomography (CT) and CT Enterography (CTE): CT scans provide detailed cross-sectional images of the small intestine, aiding in assessing disease location, extent, and complications. They are particularly useful in identifying strictures, abscesses, and fistulas. They are commonly used in patients who present as an emergency due to its ease, short scanning time, and wide availability. (Sturm, Maaser et al. 2019) **Figures 1-6 and 1-7** are examples of CTE images in IBD.

Magnetic Resonance Imaging (MRI): MRI with contrast agents can be very helpful in visualising bowel inflammation and structural abnormalities. MR enterography (MRE) provides excellent soft-tissue contrast. MRI of the pelvis is frequently used to exclude pelvic collections and essential for classification of fistulae. (Sturm, Maaser et al. 2019) **Figures 1-8 and 1-9** are examples of MRE images in IBD.

Ultrasonography: Abdominal ultrasonography is a non-invasive modality that is now increasingly used as a point-of-care imaging technique by clinicians to assess disease severity. It is most used to visualise the terminal ileum and small bowel by assessing parameters like bowel thickness, wall stratification, vasculature, and lymph nodes. (Shivaji, Segal et al. 2022) It can also help detect complications like abscesses. It is more commonly used in North America and western Europe. With the advantages it brings due to ease of examination, non-invasive technique and point-of-care use-case, it is increasing in popularity as a real-time decision making tool in outpatient clinics. The limitation is the availability of expertise. It is likely to be adopted in routine care in the near future in the UK.

Wireless Capsule Endoscopy (WCE): WCE is a relatively recent invention that involves patients swallowing a small capsule with a camera that transmits images as it travels through the GI tract. It is most valuable for evaluation of small bowel which is not accessible with a conventional endoscope. (Sturm, Maaser et al. 2019)

Biomarker Panels and Genetic Testing: Emerging biomarker panels, such as serological markers (anti-Saccharomyces cerevisiae antibodies, perinuclear antineutrophil cytoplasmic antibodies) and genetic testing (NOD2/CARD15 mutations), can aid in differentiating IBD from other conditions and predicting disease behaviour. The biomarker panels have some limitations due to lack of specificity and due to minimal impact on clinical care.

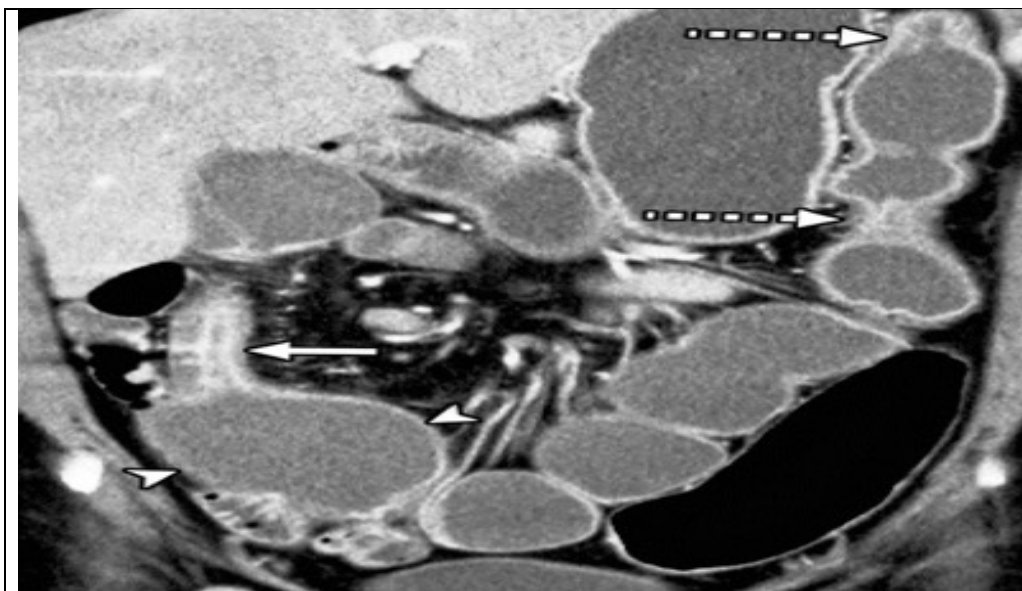


Figure 1-6 CT enterography of strictures and inflammation

Coronal contrast-enhanced CT enterography image with neutral oral contrast material shows wall thickening in the distal ileum (solid arrow) with stratified mural hyperenhancement, luminal narrowing, and moderate to severe upstream dilation (arrowheads), findings consistent with a stricture with active inflammation. There are two additional jejunal strictures (dashed arrows) with active inflammation in the left upper quadrant. (Figure adapted with permission from Guglielmo et al) (Guglielmo, Anupindi et al. 2020)

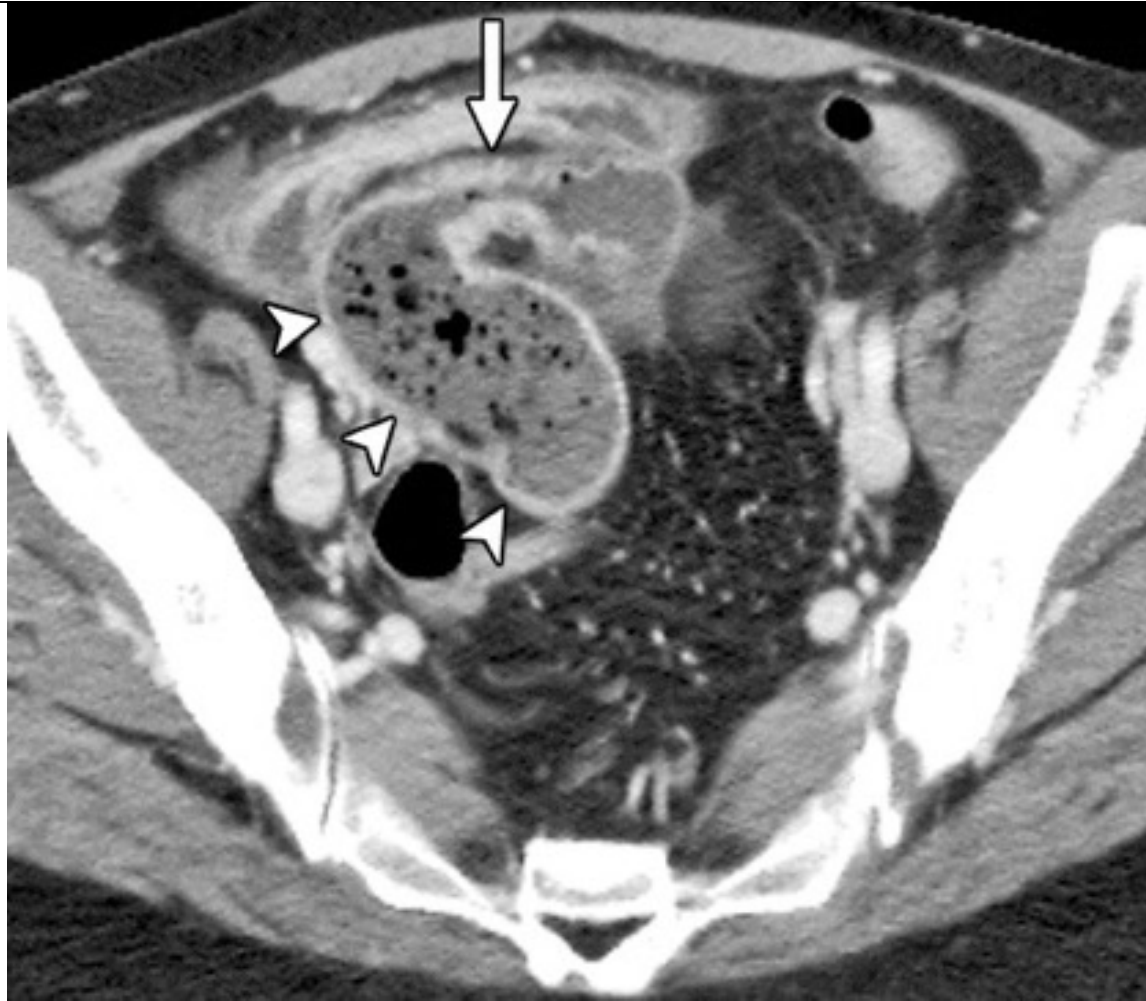


Figure 1-7 Axial view of CT enterography showing stricture in Crohn's Disease

Axial contrast-enhanced CT enterography image with neutral oral contrast material in a patient with Crohn disease shows a short segment stricture with active inflammation in the ileum (arrow), with mild upstream bowel dilation in addition to the small bowel faeces sign (arrowheads) in the dilated segment.

(Figures adapted with permission from Guglielmo et al)



Figure 1-8 MR Enterography showing simple sinus and fistula in CD

Axial T2-weighted MR enterography image with biphasic oral contrast material shows severe small bowel wall thickening and bowel wall oedema in the ileum (white arrowhead) and rectosigmoid colon (black arrowhead), findings consistent with active inflammatory Crohn disease with an associated enterocolic fistula (white arrow) and a blind-ending sinus tract (black arrow) arising from the ileum.

(Figure adapted with permission from Guglielmo et al)



Figure 1-9 MRI scan showing fibrosis and stricture

Coronal T2-weighted MR enterography image with biphasic oral contrast material shows eccentric fibrofatty proliferation (arrowheads) adjacent to a thick-walled small bowel segment (solid arrow) with luminal narrowing and mild upstream bowel dilation (dashed arrow), consistent with a stricture with active inflammation.

(Figure adapted with permission from Guglielmo et al)

1.1.4.4 Histopathology

Histopathology is an essential component for diagnosis of IBD. It is considered the gold standard in establishing the diagnosis, classification of disease and is relied upon throughout the disease journey of the patient. It is known that the initial diagnosis of UC or CD may not be apparent the first time based on histology alone and it can evolve over time.(Magro, Langner et al. 2013) The accuracy of histology requires multiple sampling, careful tissue handling and expert analysis.(Villanacci, Reggiani-Bonetti et al. 2021)

Histology is discussed in greater detail as it forms an important part of the thesis.

A normal sample of intestinal tissue is analysed systematically using four parameters and the diagnosis of IBD is made based on certain characteristic changes. The parameters used are -

Mucosal architecture

Normal terminal ileal mucosa is characterized by the ratio between the height of the villi and of the crypts, which is always in favour of the villus (2:1 or more) and normal number of intraepithelial T lymphocytes in the villi is fewer than 4 per 100 of the enterocytes.

The normal colonic mucosa is exemplified by the “test tubes in a rack” analogy: a flat luminal surface with crypts that are parallel, straight, homogeneously sized, regularly spaced, and with the bottom reaching the muscularis mucosae. No more than 10% of the crypts should be branching.(Villanacci, Reggiani-Bonetti et al. 2021)

Superficial epithelial features

The superficial epithelium is composed of absorptive and goblet cells located in a continuous layer covering the basement membrane, without any interruption or erosion.

Epithelial cellular components

In the epithelial layer, paneth cells are observed in the right and transverse colon. In the upper and middle third of the crypts, occasional apoptosis can be seen; in the lower third, mitoses are common, and immature cells can sometimes be seen.

Lamina propria cellularity

In this layer, normally lymphocytes and plasma cells are present in the lamina propria. In the right colon, eosinophils are normally present. Occasionally, primary and secondary lymphoid follicles can be observed extending to the submucosa.

Table 1-1 gives details of the features seen on histology in UC and CD. **Figures 1-10 and 1-11** illustrate features of UC and CD respectively.

Table 1-1 Histological parameters used, and general features seen in UC and CD on biopsy

Parameters	• Features in UC	• Features in CD
Mucosal architecture	• Crypt architectural abnormalities • Crypt distortion where crypts are no longer parallel • Crypt branching of muscularis mucosae • Crypt atrophy mucosae • Distorted crypt architecture	• Irregular villous architecture in ileal biopsy • Discontinuous inflammatory infiltrate is most frequent • Aphthous ulcers
Superficial epithelial features	• Surface epithelial damage • Flattening, focal cell loss • Erosions and ulcers reflect disease activity • Mucin depletion is defined as reduction in the number of goblet cells	• Crypt distortion • Crypt atrophy is less pronounced than in UC with variable distribution.

Epithelial cellular components	• Paneth cell metaplasia in the left colon • Inflammatory polyps • Hypertrophy of the muscularis mucosae • Submucosal fibrosis is less frequent but additional features of chronicity	• Focal or discontinuous changes • Pyloric gland metaplasia seen and is related to mucosal ulceration and repair
Lamina propria cellularity	• Basal plasmacytosis - plasma cells predominantly observed between the base of the crypts and muscularis mucosae • Neutrophils-reflect disease activity • Cryptitis • Crypt abscesses (presence of neutrophils within crypt lumina). • Granulomas are generally not observed	• Focal (discontinuous) chronic inflammation of variable density throughout the biopsy specimen, not confined to the superficial mucosa is characteristic of CD. • One or more circumscribed foci of increased mononuclear cell density with or without infiltration of neutrophils on a background of normal mucosa • Epithelioid cell granuloma, a discrete collection of at least five epithelioid cells, with or without multinucleated giant cells • Non-caseating granulomas, small collections of epithelioid histiocytes with giant cells or isolated giant cells • Neural hypertrophy • Infiltration of eosinophils or T lymphocytes into the submucosal or intramuscular plexus (ganglionitis) seen on surgical specimens

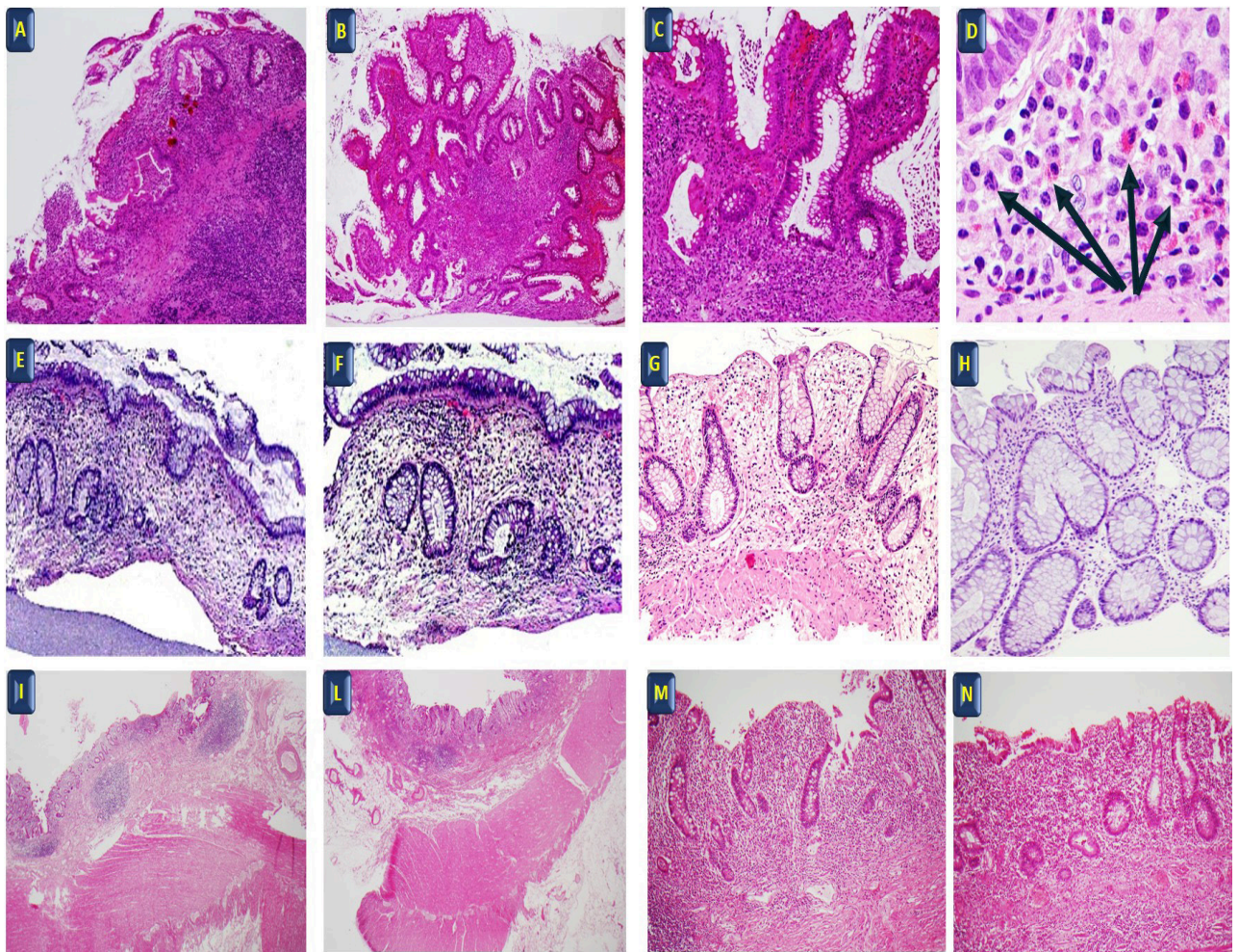


Figure 1-10 Histological features of UC

(A, B, C) Ulcerative colitis with moderate-severe activity. (D) Basal plasmocytosis with plasma cells and eosinophils (arrows). H&E A-B: 4x, C: 20x, D: 60x. (E, F, G, H) Ulcerative colitis in remission (quiescence of disease) H&E E: 10x, F-G-H: 20x. (I, L, M, N) Ulcerative colitis: surgical specimen with inflammation located in the mucosa. H&E I-L: 4x, M-N: 20x

(Figure adapted with permission from Villanacci et al)

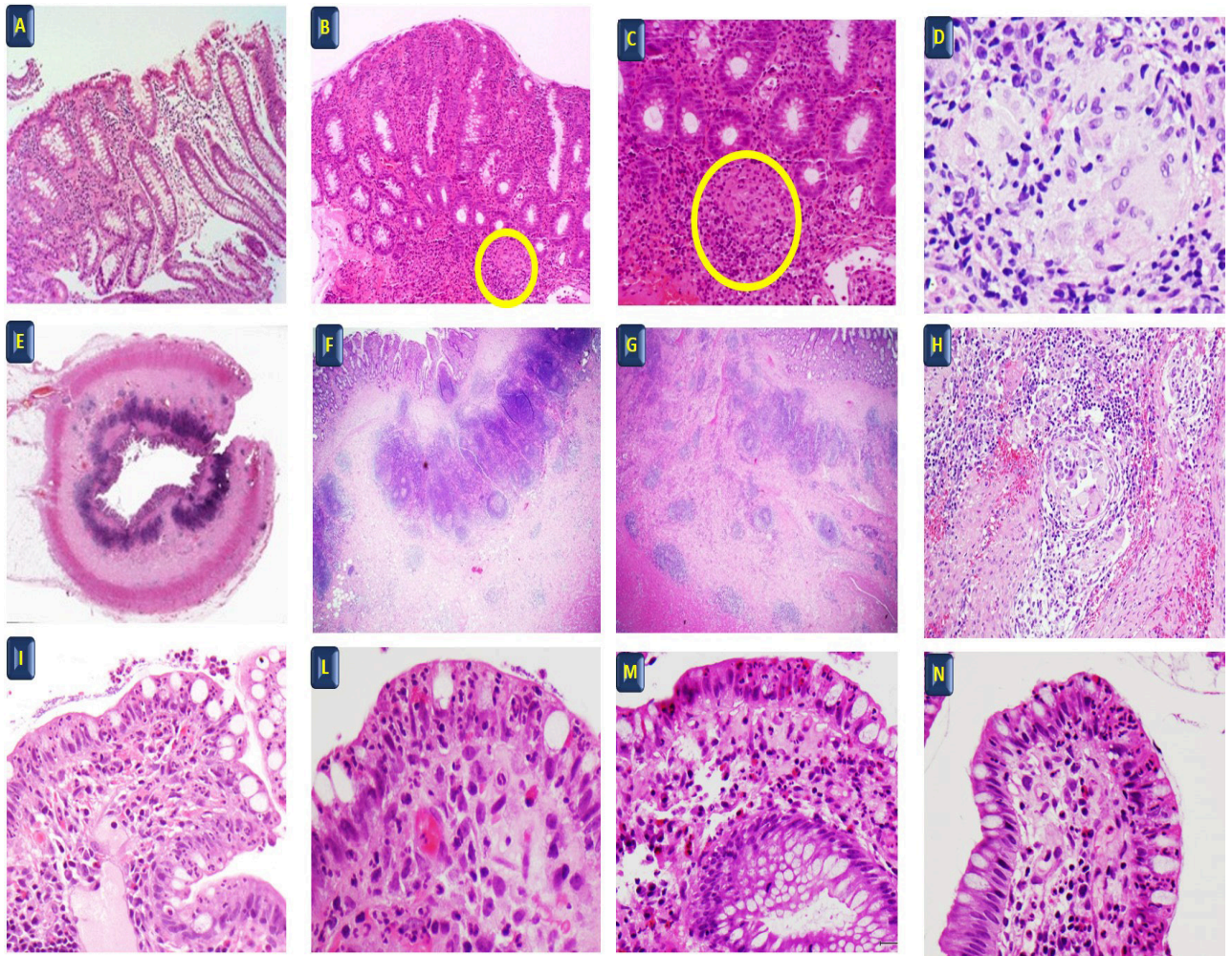


Figure 1-11 Histological features of Crohn's disease

(A) Patchy inflammation, (B-C) granuloma, (D) granuloma. H&E A-B: 20x, C: 40x, D: 60x. (E, F, G, H) Crohn's disease surgical specimen, inflammation penetrating the different layers of the terminal ileum H&E E: 4x, F: 10x, G: 20x, H: 40x. (I, L, M, N) Early Crohn's disease, focal superficial inflammation in the terminal ileum. H&E I: 10x, L: 40x, M: 20x; N 40x

(Figure adapted with permission from Villanacci et al)

Histology conveys a lot of information and can be a useful tool not only in daily clinical practice to aid decision making but also in research to get a deeper understanding of changes occurring at a tissue level. Whether it is to establish mucosa healing (Magro, Langner et al. 2013) and histological healing as targets of therapy in IBD or describe grades of dysplasia or to establish a diagnosis of cancer, histology is the tool all clinicians rely on.

1.2 Section 1B - UC and CD

1.2.1 Ulcerative Colitis

Ulcerative Colitis affects the colon and is a condition that carries significant morbidity due to the nature of symptoms. It can affect the quality of life of patients significantly. (Magro, Gionchetti et al. 2017) The incidence of UC has increased globally including in the newly industrialised countries. Although the incidence has stabilised in the western nations which are some the highest prevalence areas, the low- and middle-income countries show an increasing trend. The incidence and prevalence of UC globally based on current epidemiological data is illustrated in **Figure 1-12**. (Ng, Shi et al. 2018) As seen in the map, there are large areas where data is unavailable or is sparse.

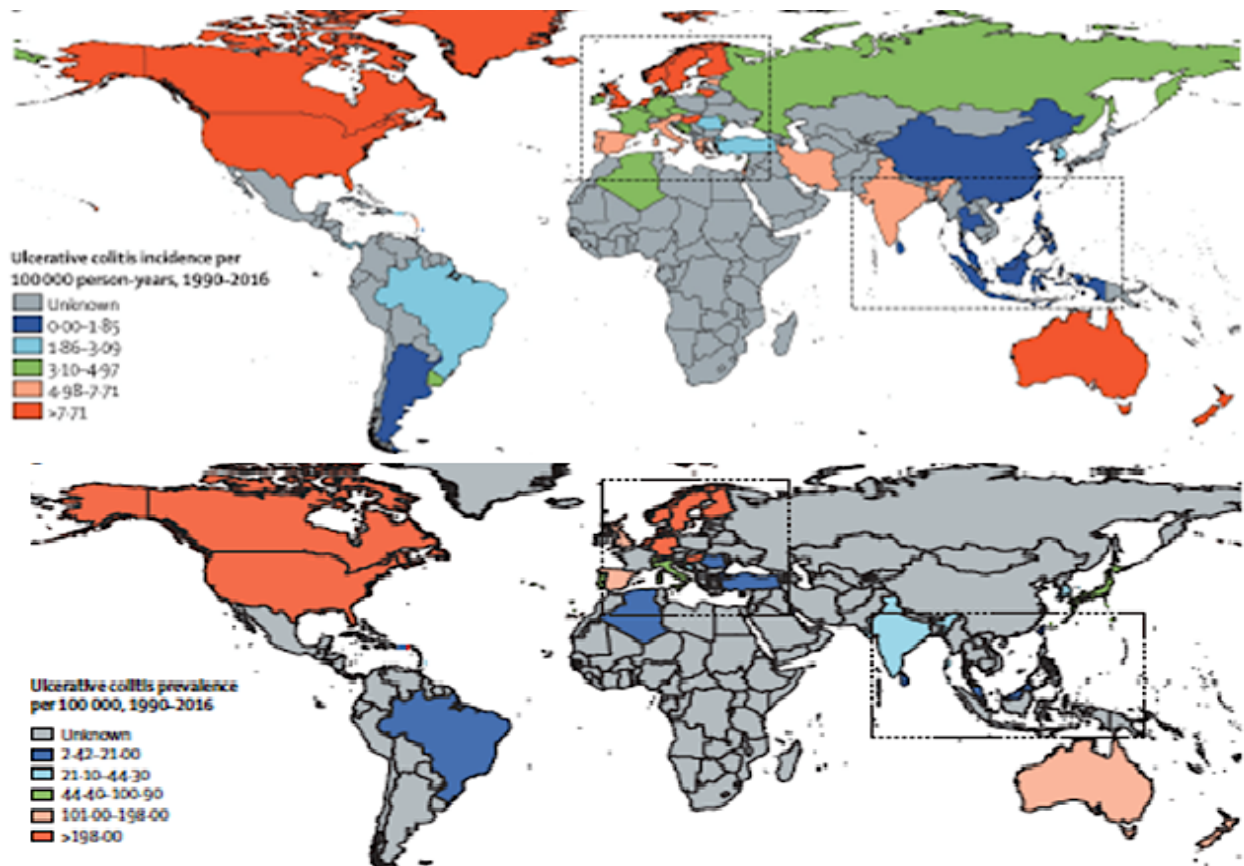


Figure 1-12 Map of incidence and prevalence of Ulcerative colitis

This map illustrates the incidence and prevalence of Ulcerative colitis per 100000 person-years from 1990 to 2016. *(Figure adapted with permission from Ng et al)*

UC is commonly classified using the Montreal Classification system, described in **Table 1-2**. This classification is currently used worldwide as a basis for guidelines by most societies. (Satsangi, Silverberg et al. 2006).

Table 1-2: Montreal classification of extent and severity in Ulcerative Colitis (UC)

Extent			Anatomy
E1	Ulcerative proctitis		Involvement limited to the rectum (that is, proximal extent of inflammation is distal to the rectosigmoid junction)
E2	Left sided UC (distal UC)	UC	Involvement limited to a proportion of the colorectum distal to the splenic flexure
E3	Extensive (pancolitis)	UC	Involvement extends proximal to the splenic flexure
Severity			Definition
S0	Clinical remission		Asymptomatic
S1	Mild UC		Passage of four or fewer stools/day (with or without blood), absence of any systemic illness, and normal inflammatory markers (ESR)
S2	Moderate UC		Passage of more than four stools per day but with minimal signs of systemic toxicity
S3	Severe UC		Passage of at least six bloody stools daily, pulse rate of at least 90 beats per minute, temperature of at least 37.5°C, haemoglobin of less than 10.5 g/100 ml, and ESR of at least 30 mm/h

ESR- erythrocyte sedimentation rate

There are few prospective population-based epidemiological studies available in literature for adult inception patients in UC. One of the most comprehensive studies recently reported is from the Epi-IBD inception cohort delivered by ECCO Epi-IBD group in Europe, one of the highest prevalence areas for IBD. This study prospectively included inception cohort of unselected IBD patients to investigate the occurrence, disease course & prognosis, with an aim also to evaluate

the disease course of UC and impact of treatment choices during the first 5 years following diagnosis. In this study, the investigators reported several aspects such as predictors of progression of disease and management trends.

Disease course in UC

One way of describing disease course is based on comparative mapping of extent during initial diagnosis to respective longitudinal follow up period. In UC, extent largely remains stable with a relatively small proportion of patients progressing further than at diagnosis and also some patients showing regression with effective management. In the Epi-IBD study, the authors reported that 61% of patients had limited UC [E1, proctitis or E2, left-sided colitis], and during follow-up about 21% experienced a progression in extent. **(Figure 1-13)** (Burisch, Katsanos et al. 2019) They noted that median time to progression was 25 months [IQR: 12–28] for E1 to E2, 17 months [IQR: 8–38] for progression from E1 to E3, and 12 months [IQR: 7–22] for progression from E2 to E3. There were no statistically significant geographical differences found in both criteria. (Burisch, Katsanos et al. 2019)

When considering regression, the authors noted that 25% regressed from E3 to either E1 or E2 after a median of 41 months [IQR: 17–50] after diagnosis. Overall, of patients with extensive colitis either at diagnosis or during follow-up, 27% regressed to a more limited disease extent **(Figure 1-13)** and regression was associated with a lower risk for all outcomes. (Burisch, Katsanos et al. 2019)

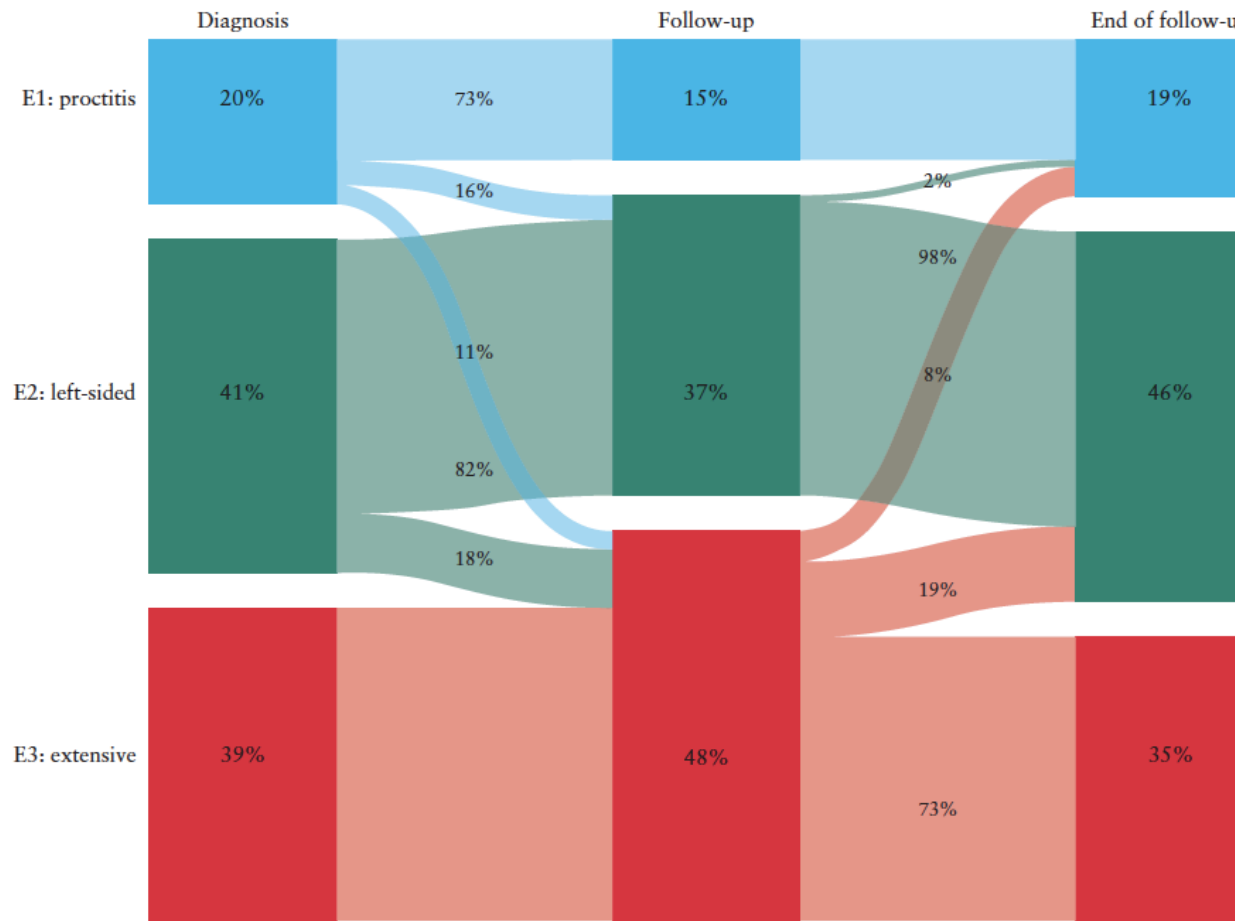


Figure 1-13 Disease extent and changes in extent in ulcerative colitis patients during 5 years of follow-up in a European population-based inception cohort

(Figure adapted with permission from Burisch et al)

1.2.2 Management of UC

The medical management of UC has evolved over decades. Although aminosalicylates remain the mainstay of treatment for mild disease activity, the availability of biologics has changed the landscape of medical management when there is more severe disease or progression with increased inflammatory burden.

Despite increasing use of biologic drugs, some patients progress or do not respond to medical therapy and therefore the use of surgical options remains an important and effective one.

1.2.2.1 Medical management of UC

Aminosalicylates: Mesalazine or mesalamine is commonly used as first-line treatment for mild-to-moderate UC. They work locally within the lumen of the colon by reducing inflammation.(Raine, Bonovas et al. 2022) These medications are generally well-tolerated and can effectively induce and maintain remission in many patients. ECCO recommends using a dose of >2grams/day for purposes of maintaining remission. (Raine, Bonovas et al. 2022) However, their effectiveness in more severe cases of UC is limited.

Corticosteroids: Corticosteroids, such as prednisone and budesonide (MMX), are potent anti-inflammatory drugs used to treat moderate-to-severe UC flare-ups. They can provide rapid relief of symptoms and induce remission. However, due to their significant side effects and limited long-term use, corticosteroids are typically reserved for short-term therapy or as a bridge to other treatments.(Raine, Bonovas et al. 2022)

Immunomodulators: Immunomodulatory drugs, such as azathioprine and 6-mercaptopurine, are used to maintain remission only and not recommended in induction of remission.(Raine, Bonovas et al. 2022) They are considered second-line treatment in UC and reduce the need for corticosteroids. These medications work by suppressing the immune system to decrease inflammation. While they have shown efficacy in UC treatment, they may take several months to exert their full effect and require regular monitoring of blood counts and liver functions.

Advanced immunological therapies

Biologic therapies

Biologics such as anti-tumour necrosis factor alpha (TNF alpha) drugs (e.g., infliximab, adalimumab, golimumab), anti-integrin agents (e.g., vedolizumab) and IL-12/23 agents (ustekinumab), have revolutionized the management of moderately-to-severely active UC.(Raine, Bonovas et al. 2022) These medications specifically target key molecules involved in the inflammatory process.

Anti-TNF alpha group of drugs remain the most frequently used first-use biologic in IBD due to years of experience, large available data, and familiarity. They are generally used after failure of first- and second-line treatments. With cumulative experience using anti-TNF drugs, there are a large number of adverse events that have been reported also. They can range from mild to life threatening events and require careful monitoring. With growing experience, clinicians are able to manage them with guidance and vigilance.(Shivaji, Sharratt et al. 2019) The more recently approved biologics have demonstrated reasonable rates of induction of remission and maintenance and probably have a better safety profile compared to anti-TNF alpha group of drugs.

Small molecule oral targeted therapies

Orally absorbed small molecules have attracted interest as a new class of oral targeted therapies because of the ease of oral administration and because this route of administering medicines is generally considered to be conventional and familiar to patients. (Shivaji, Nardone et al. 2020)

There are multiple targets currently being interrogated. Some of these have been successful and are currently being exploited for treatment of IBD, and others are likely to be available in the near future. (Shivaji, Nardone et al. 2020) **Figure 1-14** illustrates the molecular targets and drugs.

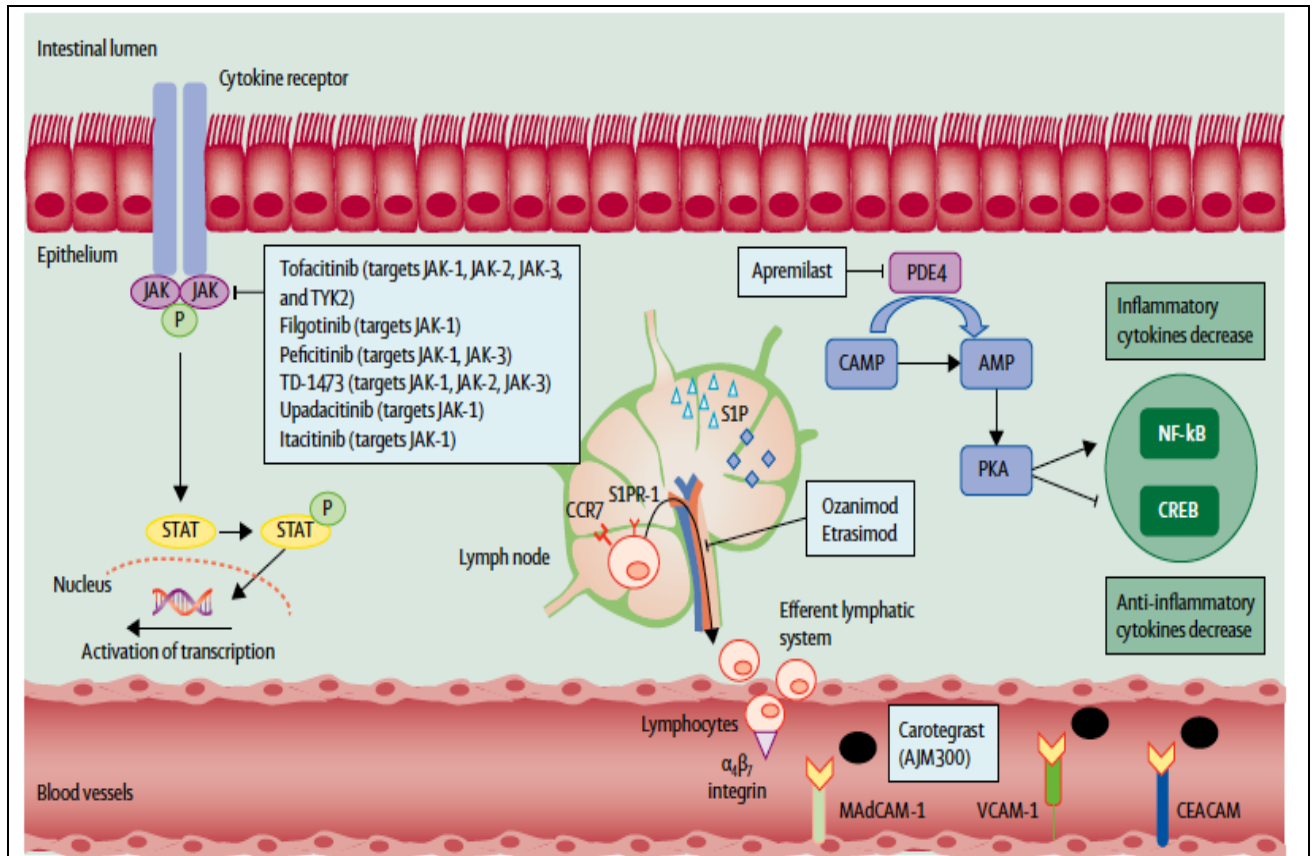


Figure 1-14 Targets and oral targeted therapies for IBD

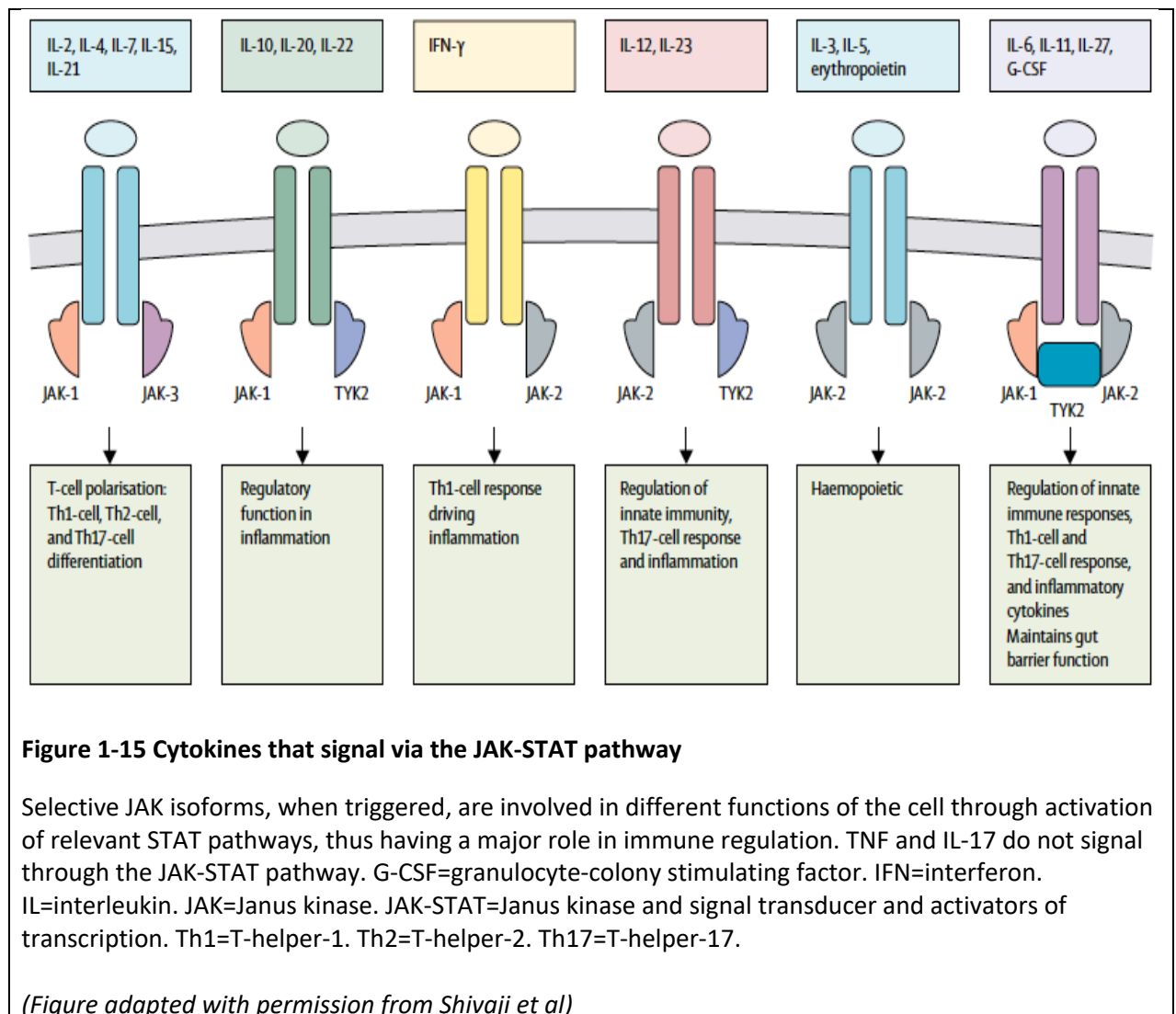
cAMP=cyclic AMP. CCR7=C-C chemokine receptor 7. CEACAM=carcinoembryonic antigen-related cell adhesion molecule 1. CREB=cAMP response element-binding protein. JAK=Janus kinase. JAK-STAT=Janus kinase and signal transducer and activators of transcription. MADCAM-1=mucosal addressin cell adhesion molecule 1. NF-κB=nuclear factor kappa-light-chain-enhancer of activated B cells. PDE4=phosphodiesterase=4. P=phosphorylation of the isoforms. PKA=protein kinase A. S1P=sphingosine-1-phosphate. S1PR-1=sphingosine-1-phosphate receptor 1. STAT=signal transducer and activators of transcription. TYK2=tyrosine kinase 2. VCAM-1=vascular cell adhesion protein 1.

(Figure adapted with permission from Shivaji et al)

Janus Kinase Inhibitors: Janus kinase (JAK) inhibitors are newly approved group of drugs in IBD.

These are oral medications that block the JAK-STAT pathway involved in inflammation. As they

exert their action at the receptor level, they are able to block the effects of multiple pro-inflammatory cytokines instead of a single target.(Shivaji, Nardone et al. 2020) **Figure 1-15** illustrates the JAK receptors and the cytokines blocked. Tofacitinib, filgotinib and upadacitinib are examples of JAK inhibitors. These are currently approved for treatment of moderately-to-severely active UC.(Danese, Vermeire et al. 2022) As they are relatively new drugs, we need more experience in using them effectively and managing the adverse events.



Novel oral targeted therapies: Several other emerging therapies are being investigated (Figure 1-9), such as sphingosine-1-phosphate receptor modulators (e.g., ozanimod and etrasimod),

phosphodiesterase inhibitors and oral anti-integrins. They are in clinical trials and not yet approved for therapy.

Throughout their disease journey, patients with UC require a range of therapies to keep them in remission, based on multiple factors. A representation of the cumulative probability of first-drug prescription during the first five years of diagnosis is given in **Figure 1-16**. (Burisch, Katsanos et al. 2019)

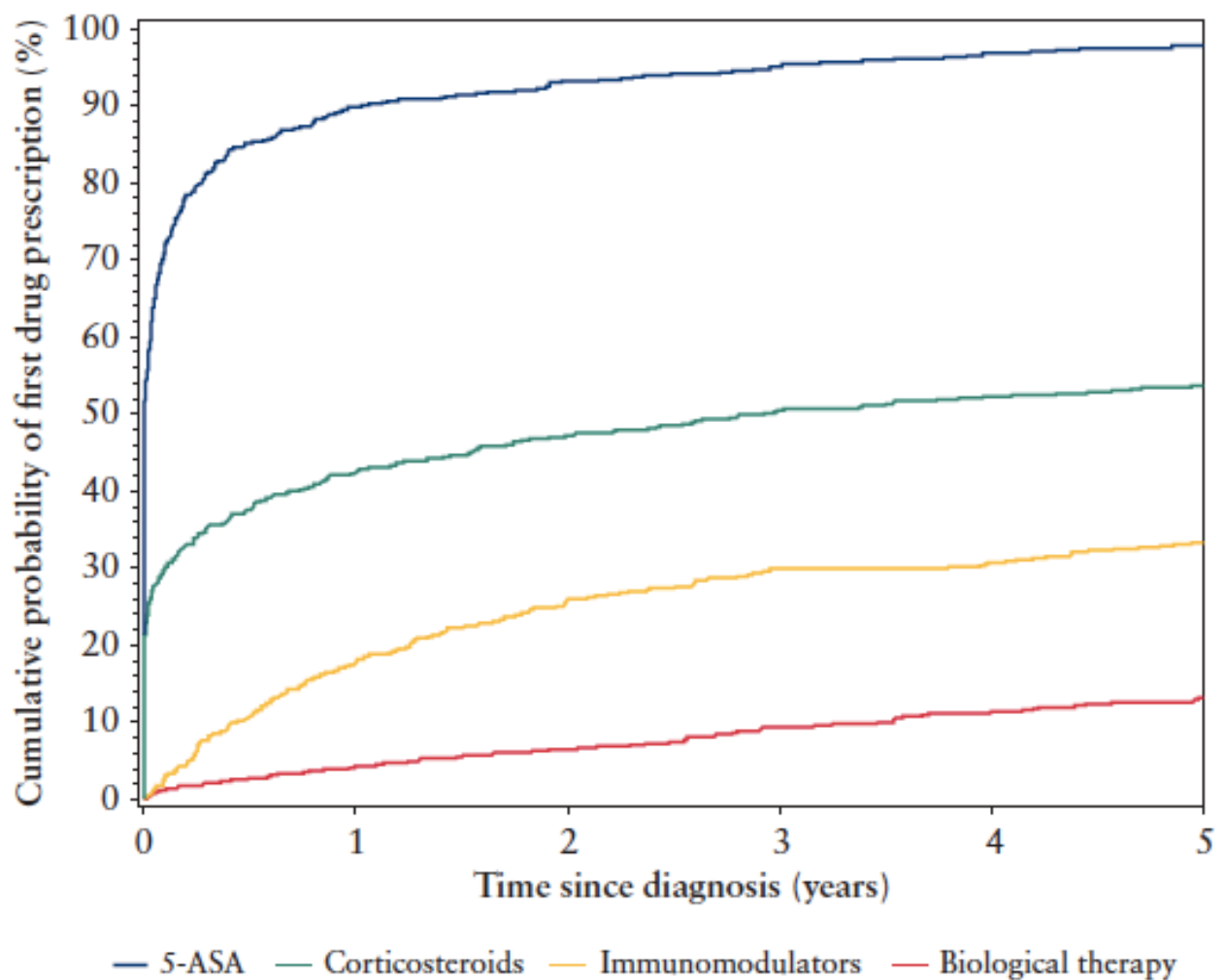


Figure 1-16 Cumulative 5-year exposures for medical treatment of patients with ulcerative colitis in a European inception cohort for the entire cohort

(Figure adapted with permission from Burisch et al)

1.2.2.2 Surgical management of UC

While medical therapy remains the primary treatment approach, surgical intervention plays a crucial role in the management of UC, especially for patients with severe disease, medication failure, or complications. It offers curative options and improved quality of life for patients who have failed medical therapy and for patients who present with acute fulminant colitis with life threatening signs. The decision to use surgical options depends on patient profile, urgency and patient choices. ECCO recommends a laparoscopic approach as it is associated with lower intra- and postoperative morbidity, faster recovery, fewer adhesions and incisional hernias, shorter hospital length of stay, improved female fecundity, and better cosmesis. (Spinelli, Bonovas et al. 2022)

Colectomy

Colectomy is the most definitive treatment for UC. It is performed either as a total proctocolectomy (removal of the entire colon and rectum) or a subtotal colectomy, where the entire colon is resected except the rectum, thereby leaving behind a rectal stump. The decision regarding the extent of colectomy depends on factors such as disease severity, extent, patient preferences, and surgeon expertise.

Restorative Procedures

Ileal Pouch-Anal Anastomosis (IPAA): IPAA, also known as the J-pouch procedure, involves creating a reservoir from the small intestine (ileum) and connecting it to the anus. This procedure offers a restoration of bowel continuity, preservation of anal function, and elimination of the need

for a permanent stoma. It is usually done as a modified 2-stage procedure with favourable results. (Spinelli, Bonovas et al. 2022)

Ileoanal Reservoir with Diverting Ileostomy: In cases where IPAA is not feasible or contraindicated, ileoanal reservoir may be performed. This procedure involves creating an internal reservoir using the ileum, which is then connected to a temporary ileostomy. After a period of healing and assessment, the ileostomy is reversed, allowing for continence and improved quality of life. IRA has better functional outcomes for patients compared to IPAA. (Spinelli, Bonovas et al. 2022)

Other Surgical Options

Proctocolectomy with Permanent End Ileostomy: This procedure involves the removal of the entire colon and rectum, with the creation of a permanent stoma. Although it eliminates the risk of pouch-related complications, it may have a significant impact on body image and psychological well-being. However, it remains a viable option for select patients, particularly those with extensive disease or medical comorbidities.

Colectomy with ileorectal anastomosis (IRA): In cases where UC is limited to the colon and does not involve the rectum, colectomy with IRA can be considered. This procedure involves removing the colon while preserving the rectum and connecting the small intestine directly to the rectum. However, it may not be suitable for patients with a high risk of rectal cancer or severe rectal inflammation. (Spinelli, Bonovas et al. 2022)

The surgical outcomes are generally favourable, with a significant improvement in symptoms, quality of life, and long-term disease control. However, potential complications such as pouchitis, anastomotic leaks, sexual dysfunction, and infertility should be discussed with patients during preoperative counselling.

1.2.3 Crohn's Disease

Crohn's disease (CD) carries significant morbidity and mortality. Recent systematic reviews have noted that over the years since its recognition, the incidence and prevalence has been increasing. (Molodecky, Soon et al. 2012, Ng, Shi et al. 2018) The incidence and prevalence of CD globally based on current epidemiological data is illustrated in **Figure 1-17**. (Ng, Shi et al. 2018) (As seen in the map, there are large areas where data is unavailable or is sparse.

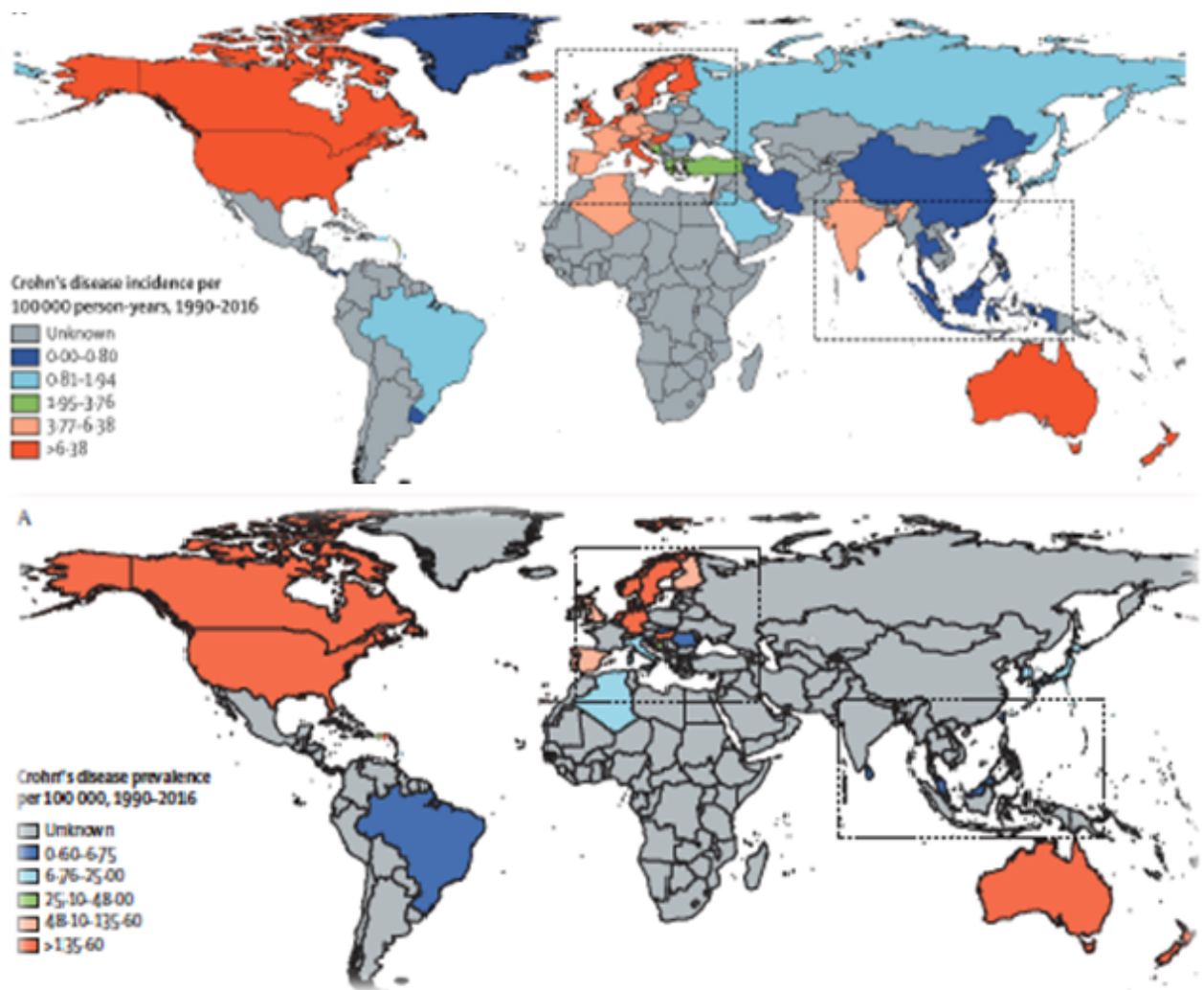


Figure 1-17 Map of incidence and prevalence of Crohn's Disease

This map illustrates the incidence and prevalence of Crohn's Disease per 100000 person-years from 1990 to 2016. (Figure adapted with permission from Ng et al

Disease course in CD

The disease course with CD can be quite variable and, in most cases, aggressive. As per long term population-based data (1935–2008), 50% of adult patients with CD have an intestinal complication within 20 years of diagnosis, with a 20% annual incidence rate of hospital admission. About 50% of patients undergo surgery within 10 years after diagnosis and the risk of postoperative recurrence is 44–55% after 10 years.(Baumgart and Sandborn 2012) Some high risk features that predict a disabling disease course include patients who are diagnosed at a younger age, immediate need for corticosteroids, perianal disease and stricturing phenotype.(Baumgart and Sandborn 2012) Only 10% of patients have prolonged clinical remission.

CD is classified using the Montreal classification, as described in **Table 1-3**. As per some population based studies, at initial diagnosis, terminal ileal disease (L1) (**Figure 1-18**) was most common and most patients had a non-stricturing non-penetrating phenotype (B1).(Baumgart and Sandborn 2012)

Table 1-3 Montreal classification for Crohn's Disease

Montreal classification for Crohn's Disease	
Age at diagnosis	
A1	Onset below 16 y
A2	Onset between 17 and 40 y
A3	Onset above 40 y
Location	
L1	ileal
L2	colonic
L3	ileocolonic
L4	isolated upper disease*
Behaviour	
B1	non-stricturing, non-penetrating
B2	stricturing

B3	penetrating
	p perianal disease modifier†

*L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present.

†“p” is added to B1–B3 when concomitant perianal disease is present.

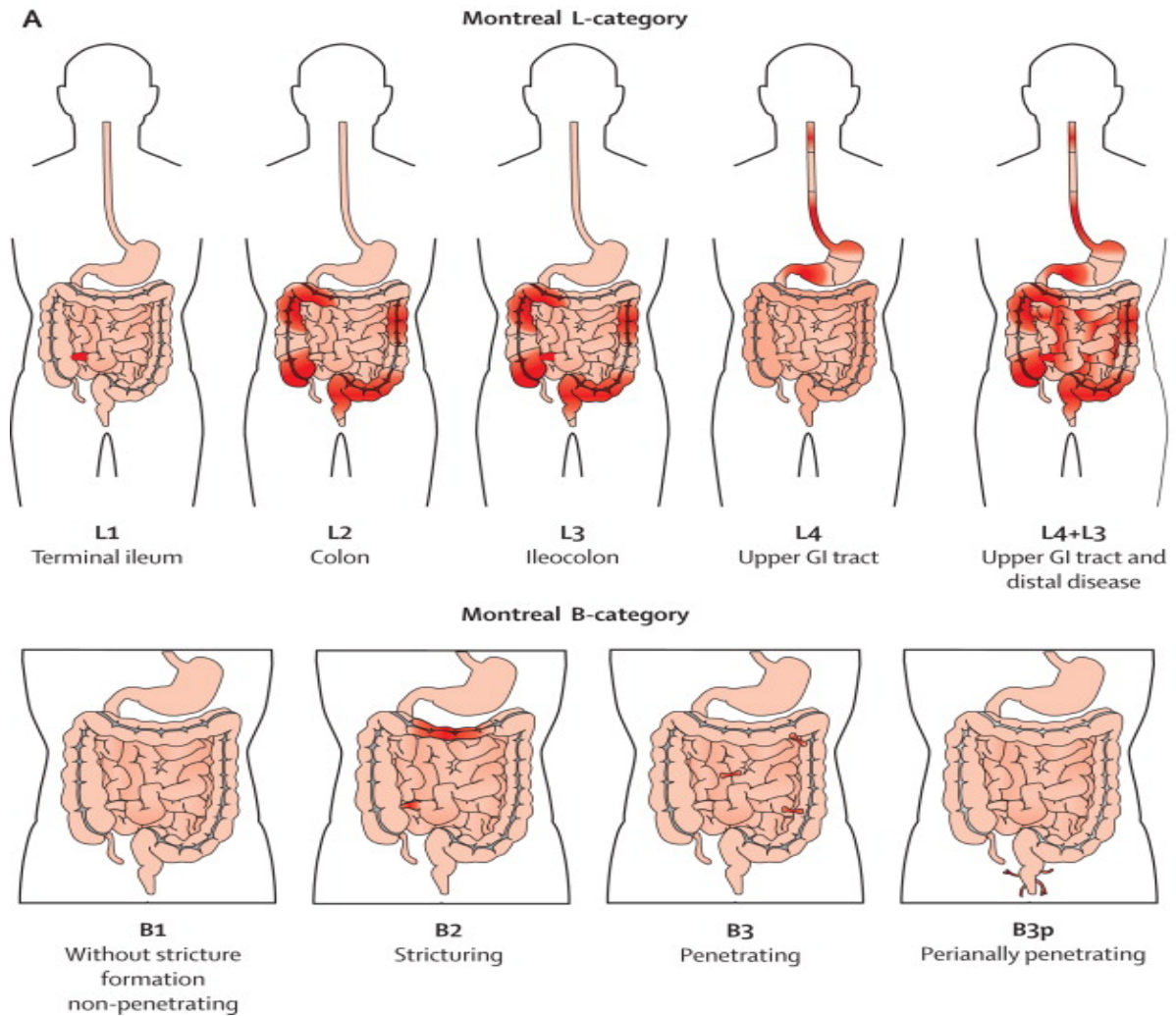


Figure 1-18 Montreal classification of CD for location(L) and behaviour(B)

(Figure adapted with permission from Baumgart et al (Baumgart and Sandborn 2012))

There are few prospective population-based epidemiological studies available in literature for adult inception patients in CD. In one of the largest recent prospective epidemiological studies, the Epi-IBD study covering a large geographical area, patients were prospectively recruited from multiple centres in Europe. (Burisch, Kiudelis et al. 2019)

The investigators included 476 patients at diagnosis and followed them up longitudinally for 5 years gathering comprehensive data regarding demographics, disease activity, medical therapy, including dose, date of initiation and date of cessation, surgery, hospitalisation, disease classification, cancers and deaths. As part of this study, they also mapped the disease course of patients in the first 5 years of natural history of CD. In this cohort, majority of the patients had active disease at diagnosis. There was an increase in proportion of patients in remission from 33% in the first year to during to 77% in the fifth year of follow-up, as illustrated in **Figure 1-19**. (Burisch, Kiudelis et al. 2019)

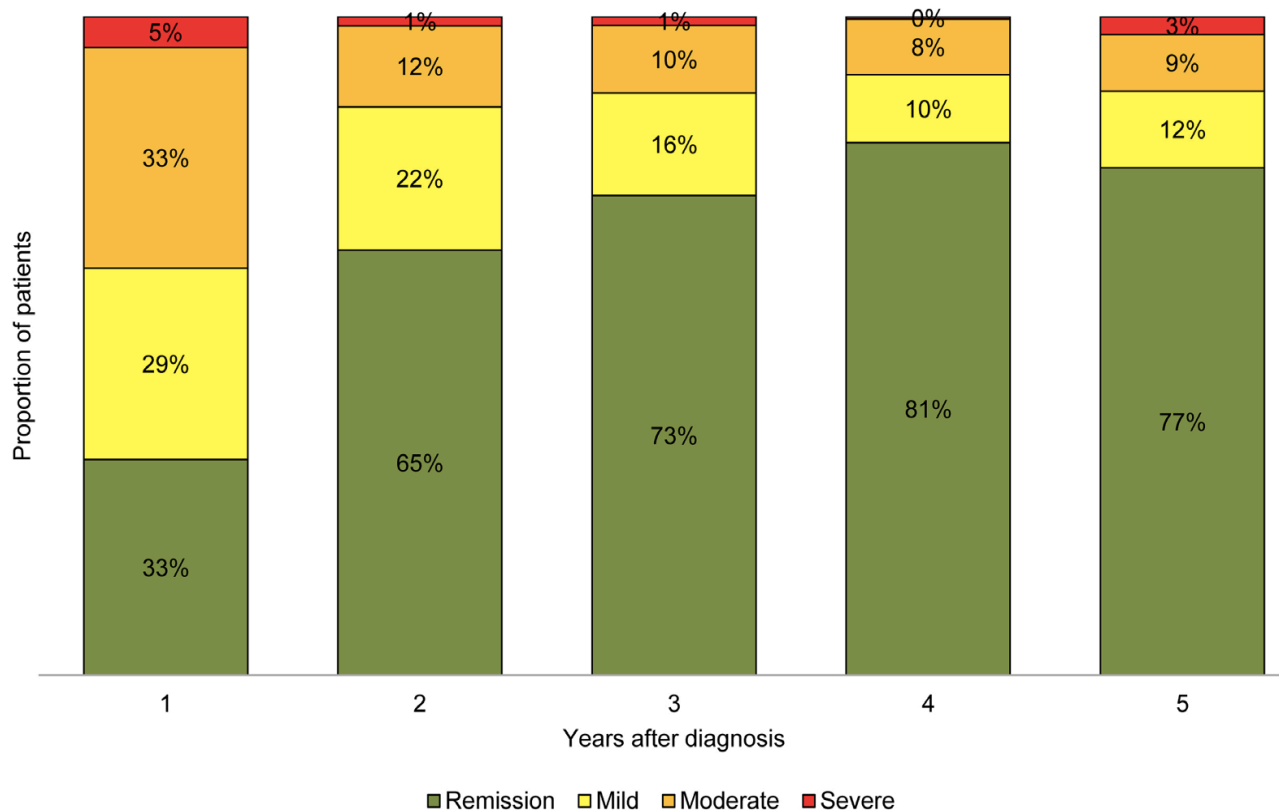
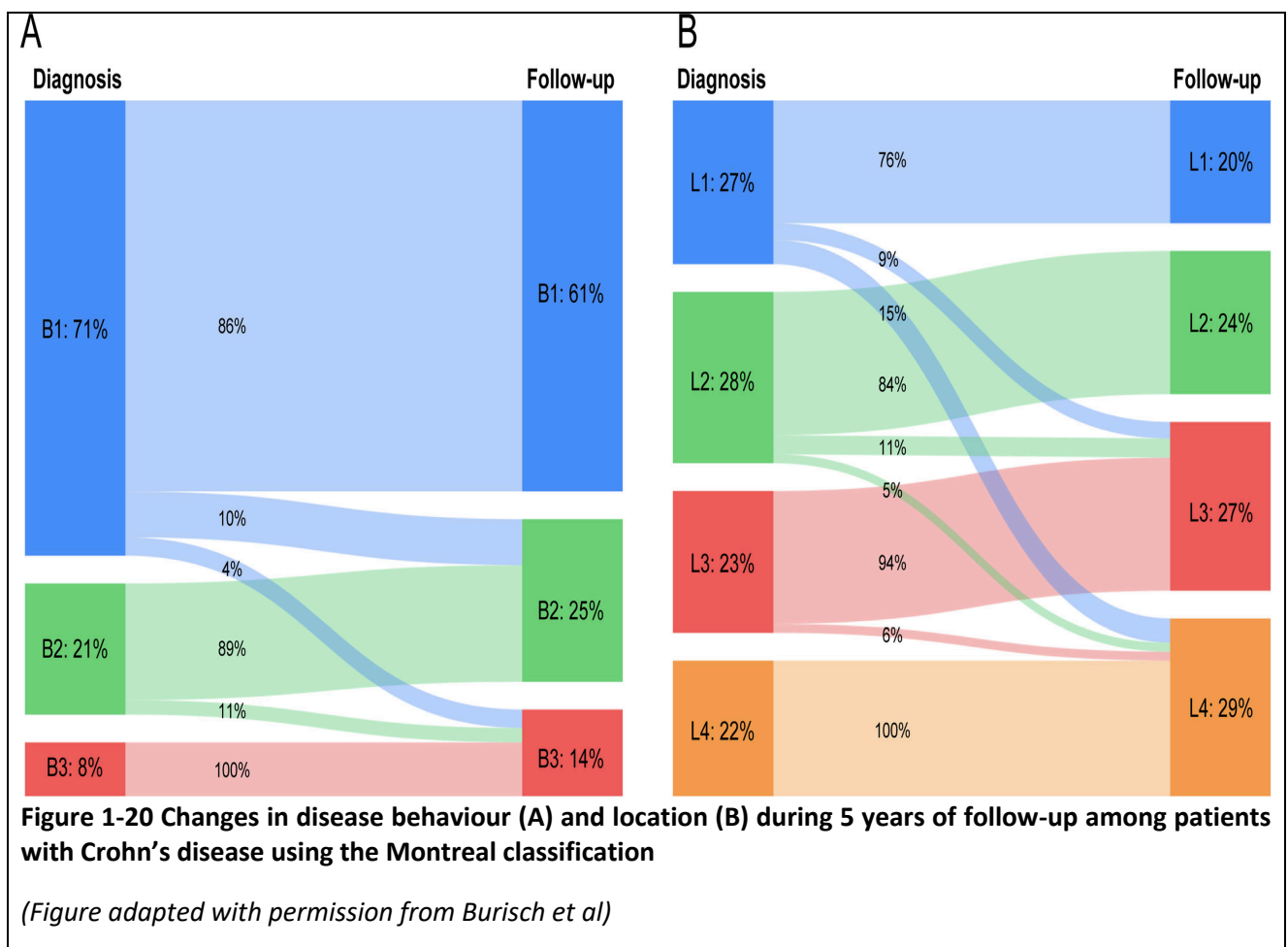


Figure 1-19 Distribution of disease activity among patients with Crohn's disease during 5 years of follow-up

(Figure adapted with permission from Burisch et al)

In terms of disease location, the investigators reported a progression observed in about 12% of patients, with a median time to any change in location of 13 months (IQR 8–41). **(Figure 1-20)**

In terms of disease behaviour, about 29% patients had complicated CD at diagnosis which increased to 39% at 5 years. Overall, 14% of patients diagnosed with B1 progressed to either B2 or B3 and 11% progressed from B2 to B3. **(Figure 1-20)**



Majority of patients in this cohort received treatment at diagnosis, with about 30% receiving biologics during the 5 year follow-up. The burden of hospitalisation in CD was quite significant. Overall, 36% of patients were hospitalised and 23% during the first year. The median time to first hospitalisation was 6 months (IQR 1–22).

1.2.4 Management of CD

Treatment of CD aims to achieve sustained clinical and endoscopic remission (mucosal healing) and interrupt the naturally progressive destructive disease. Patients with CD are more likely to receive immunomodulators and biologics to manage their disease. Only about 3% of patients in the epidemiological study by Burisch et al did not receive any treatment during 5 years of follow up. **Figure 1-21** illustrates the cumulative drug exposure in this cohort. (Burisch, Kiudelis et al. 2019)

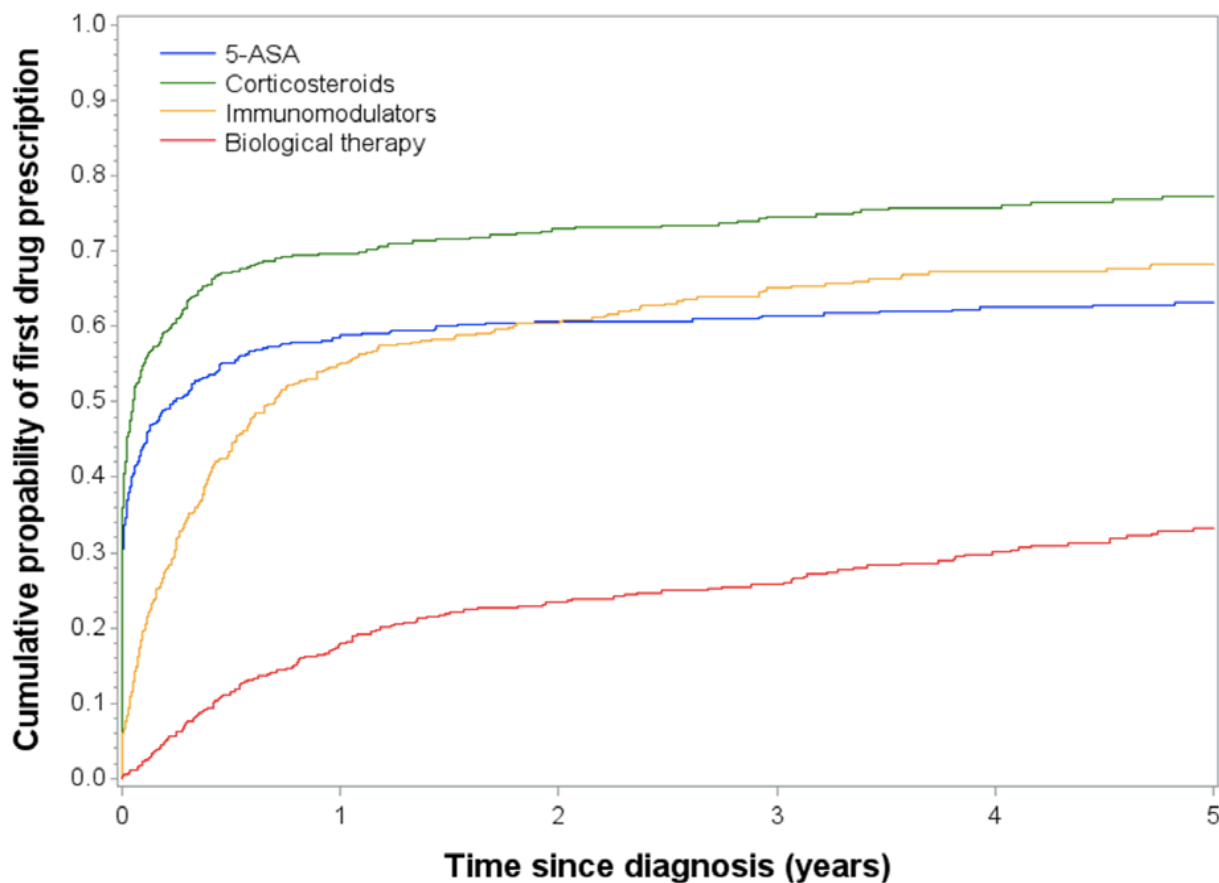


Figure 1-21 Cumulative 5-year exposures for medical treatment for patients with Crohn's disease in a European inception cohort

1.2.4.1 Medical management of Crohn's Disease

The medical management of CD is perhaps more complex than UC and depends on the location and behaviour. A brief overview of drugs used is discussed in this section.

Aminosalicylates: Aminosalicylates, such as mesalazine, continue to be used as a first-line treatment for mild Crohn's colitis. Based on current available evidence, ECCO advises against the use of mesalazine in CD. (Torres, Bonovas et al. 2020) Despite the poor evidence, it continues to be used by clinicians in patients with milder disease activity. A significant proportion of CD patients remain on mesalazine at various stages of their disease. **(Figure 1-21)** As per the Epi-IBD study group findings, about 292 (60%) patients with Crohn's disease received 5-aminosalicylate period during follow-up for a median duration of 28 months (interquartile range 6-60). (Burisch, Bergemalm et al. 2020) A large nation-wide cohort by Atia et al looked at data from 19,264 patients diagnosed with CD between 2005 and 2020. Among these, 45% fulfilled the eligibility criteria and 16% received 5-ASA and 29% received no mesalazine. They concluded that first-line 5-ASA monotherapy was not superior no mesalazine but associated with a slightly higher rates of adverse events. (Atia, Goren et al. 2023) The reasons for continued use were reported in a survey of physicians' beliefs. The perceived efficacy in subgroups of CD patients, safety, and patient preferences primarily motivated aminosalicylate prescription in CD. Physicians also reported a lack of robust evidence against the use of mesalazine in CD. (Ma, Ascoytia et al. 2018) An ongoing clinical trial called the STATIC trial which is a multicentre randomised controlled trial will likely answer this question with a degree of certainty. (STATIC 2024) The results of this study are awaited.

The role of mesalazine as a probable chemoprophylaxis medication in colorectal cancer could be one of the reasons clinicians continue to use it in patients who have extensive Crohn's colitis. (Majumder, Shivaji et al. 2022)

Corticosteroids: Corticosteroids are used to induce remission. ECCO recommend using budesonide for the induction of clinical remission in patients with active mild-to-moderate Crohn's disease limited to the ileum and/or ascending colon. In moderate-to-severe or more extensive CD, oral prednisolone or systemic steroids are recommended. (Torres, Bonovas et al. 2020). Steroids are never used as a long-term treatment strategy due to significant long term side effects.

Immunomodulators: Immunomodulators, such as thiopurines and methotrexate are commonly employed as steroid-sparing agents in CD. Due to the slow onset of action, they are not recommended as monotherapy in induction of remission. (Torres, Bonovas et al. 2020) Thiopurines can be useful in maintaining remission when patients have steroid-dependent disease with low disease burden. They are commonly used as combination therapy with specific drugs such as infliximab, as it helps lower the risk of antibody formation against this drug. (Torres, Bonovas et al. 2020) They remain popular due to low cost and ease of administration. However, their role in managing CD early in the disease is not very clear. ECCO recommends against using it early, based on data showing limited benefit and more adverse events compared to placebo. (Panés, López-Sanromán et al. 2013) The other immunomodulators such as parenteral methotrexate are not recommended for induction but can be used for maintenance of remission in some patients. (Torres, Bonovas et al. 2020)

Advanced immunological therapies

Biologic therapies

Biologic therapies such as anti-TNFs, vedolizumab and ustekinumab have demonstrated efficacy in inducing and maintaining remission, particularly in moderately-to-severely active CD. They are recommended for induction of remission, maintenance as well as in perianal disease. Commonly used as combination therapies (thiopurine+ anti-TNFs), they are now increasingly used as monotherapies assisted by therapeutic drug monitoring strategies.(Torres, Bonovas et al. 2020) Anti-TNF drugs are particularly useful in patients with perianal fistulising disease. The availability of newer group of biologics has increased the number of options available for patients and therefore a larger proportion of patients are being treated with biologics earlier in the course of the disease. (Illustrated in **Figure 1-21**)

Small molecules oral targeted therapies

Emerging small molecule targeted therapies, such as Janus kinase (JAK) inhibitors offer new treatment options for patients with refractory CD who have failed conventional therapies. These medications target specific molecules involved in the inflammatory cascade and have shown promising results in clinical trials. The most recent small molecule approved for treatment of moderately-to severely active CD is upadacitinib, which is a selective JAK-1 receptor inhibitor. (FDA 2023) Although the clinical trial results are promising, long-term safety data are still being collected, and their place in the treatment algorithm is evolving.

1.2.4.2 Surgical management of Crohn's disease

Surgical intervention in CD is indicated in patients who have failed medical therapy, in event of complications (such as strictures, fistulas, abscesses, and perforations), intestinal obstruction, dysplasia, and malignancy. Surgery is an important strategy overall in CD and its success depends on multidisciplinary approach and careful patient selection, (Adamina, Bonovas et al. 2020) but unlike UC, surgery is unfortunately not curative for CD due to recurrence of disease.

In some patients, surgery can be used very early in the diagnosis. For patients with symptomatic but limited disease (ileal or ileocaecal), primary resection is a useful option. The LIRIC trial compared ileocaecal resection versus infliximab and concluded that surgery is a reasonable alternative to infliximab therapy (Ponsioen, de Groof et al. 2017), and also a cost-effective one. (Groof, Stevens et al. 2019)

Commonly used techniques in surgery based on disease phenotype will be briefly discussed here.

Surgical Techniques

Strictureplasty: This is an effective surgical technique for managing strictures, preserving bowel length and function. Different types of strictureplasty techniques, such as Heineke-Mikulicz, Finney, and side-to-side isoperistaltic techniques are utilized based on the location and length of the strictures. Strictureplasty is recommended whenever it is technically feasible, particularly with multiple fibrotic strictures that would otherwise require more than a minimal bowel resection. (Adamina, Bonovas et al. 2020)

Bowel Resection: Segmental bowel resection is performed in cases of strictures, perforations, or refractory disease. Rarely do patients require emergency surgery unless there is evidence of

bowel ischaemia or perforation with sepsis. A planned operation after optimisation of nutrition using a laparoscopic approach as first line option is recommended where possible.(Adamina, Bonovas et al. 2020)

Perianal Disease Management: Surgical procedures for perianal CD include seton placement, abscess drainage, fistulotomy, and sphincter-sparing techniques, tailored to individual patient characteristics and fistula complexity. In patients who have chronic pelvic sepsis with perianal disease or refractory Crohn's colitis, a defunctioning stoma formation is known to be useful. (Adamina, Bonovas et al. 2020)

Post-operative care and outcomes

Surgical outcomes in CD have improved significantly over the years in general due to advances in surgical techniques, perioperative care, and enhanced multidisciplinary management. The known key factors influencing outcomes include disease location & behaviour, surgical experience, and patient-related factors. While surgery provides symptomatic relief and improves quality of life, complications such as anastomotic leaks, wound infections, recurrence, short bowel syndrome etc do occur. The technical aspects too are influenced by multiple factors such as surgeon's previous training, personal experiences, available resources, and clinical situation.

Newer evidence provided by recent studies have improved outcomes by adding to our knowledge. For example, during resection, it is now accepted that stapled small-bowel or ileocolic side-to-side anastomoses are associated with lower rates of postoperative complications than end-to-end anastomoses. (Simillis, Purkayastha et al. 2007)

Despite many advances, in the surgical field, there remain many areas where there is paucity of evidence, particularly in CD, and these require further research. Some of these dilemmas that exist have been well described in literature,(Meima-van Praag, Buskens et al. 2021) which also make interesting research questions. Some interesting dilemmas include -

- Advantages of strictureplasty over a resection in patients with stricturing disease without any complications? → as the median length of loss of small bowel remains low even with resections and both techniques have similar recurrence rates
- Advantages of total mesorectal excision over a close bowel resection during ileocolonic resection or proctectomy? → due to the creeping fat phenomenon around strictures in CD, the mesentery is considered to be immunologically active. There is conflicting evidence on recurrence rates between the two techniques and remains a topic of debate. (Meima-van Praag, Buskens et al. 2021)
- Advantages of a wide resection margin from macroscopically active disease compared to a short one? → previous studies have shown no difference in recurrence rates with either technique including when microscopic CD was present at the resection margins. (Fazio, Marchetti et al. 1996) However, this finding has been contradicted in more recent studies that show significant differences in recurrence rates. When resected margins were positive for inflammation, Poredska et al reported the risk of endoscopic recurrence at 6 months to be 26-fold higher in patients with positive than in those with negative histology. **(Figure 1-22)** On multivariate analysis positive resection margins had the greatest influence on recurrence compared to other factors. (Poredska, Kunovsky et al. 2019)

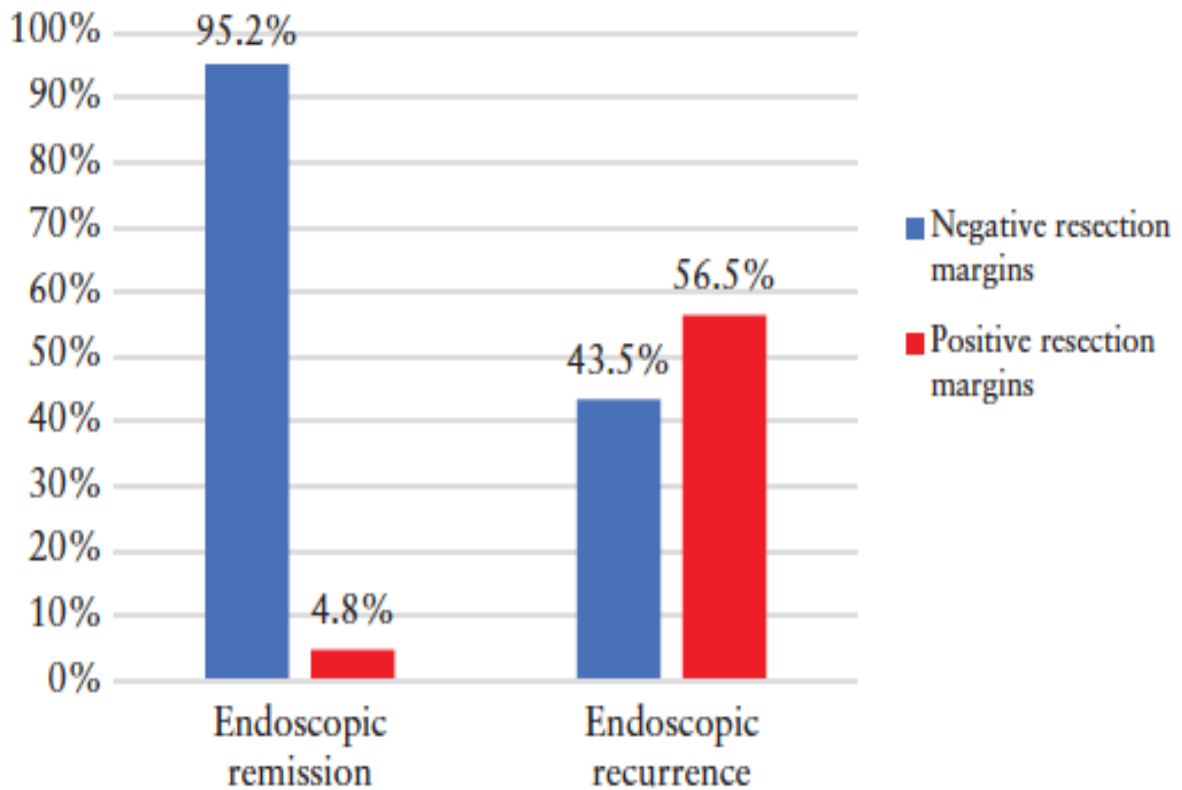


Figure 1-22 Influence of histologically inflamed resection margins on endoscopic recurrence

(Figure adapted with permission from Poredska et al)

The dilemma about resections margins is a common one and is debated by many surgeons. It is now common to use a wide margin in IBD patients who have sufficient length of bowel left. We wanted to get a deeper understanding of this at a molecular level and included it as one of the secondary objectives in our prospective study. Our results may contribute to the identification of patients with a high risk of early post-surgical endoscopic recurrence, who could benefit from more aggressive and targeted postoperative therapy.

1.3 Section 1C – Chronic inflammation in IBD

Chronic inflammation - factors, mechanisms, and complications of persistent (non-resolving) inflammation in IBD

1.3.1 Chronic inflammation or non-resolving inflammation

Inflammation is an essential process that has been evolutionarily conserved characterized by activation of immune and non-immune cells that protect the host from bacteria, viruses, toxins and infections by eliminating pathogens and promoting tissue repair and recovery. (Furman, Campisi et al. 2019) There are various degrees of inflammatory response such as systemic or local, and physiological changes occur in response to the same. This variation is also the reason why in some patients, symptoms such as fatigue, fever, reduced activity, reduced appetite, social withdrawal etc occur. These behavioural changes can be critical for survival during times of physical injury and microbial threat.(Straub 2017) This reflects an attempt to conserve energy and divert nutrients towards the inflammatory site. (Furman, Campisi et al. 2019)

In normal health, an inflammatory response is characterized by constrained upregulation of inflammatory activity when there is a threat, and resolution of this process once the threat has passed.(Furman, Campisi et al. 2019) However, there are some settings where there is not only a prevention of resolution of this acute state of inflammation, but also promotion of a state of low-grade systemic chronic inflammation characterized by the activation of immune components. These components appear to be distinct from those involved in an acute immune response. (Straub 2017) The specific reason/s for this non-resolution are unclear but could be due to various environmental, biological, and psychological conditions that have been identified as risk factors.

This process of chronic inflammation (CI) once triggered can result in loss of immune-tolerance and cause major changes at cellular level as well as significant long-term damage to tissues and organs, increasing the risk of chronic inflammatory diseases. (Kotas and Medzhitov 2015)

There are numerous causes of low-grade systemic CI that have been identified such as chronic infections, physical inactivity, obesity, intestinal dysbiosis, poor diet and exposure to antibiotics among others. In addition to above, psycho-social factors such as social isolation, psychological stress, disturbed sleep, disrupted circadian rhythm, and environmental factors such as air pollutants, hazardous waste products, industrial chemicals and tobacco smoking are implicated.

Figure 1-23 illustrates the causes and various effects of systemic chronic inflammation.

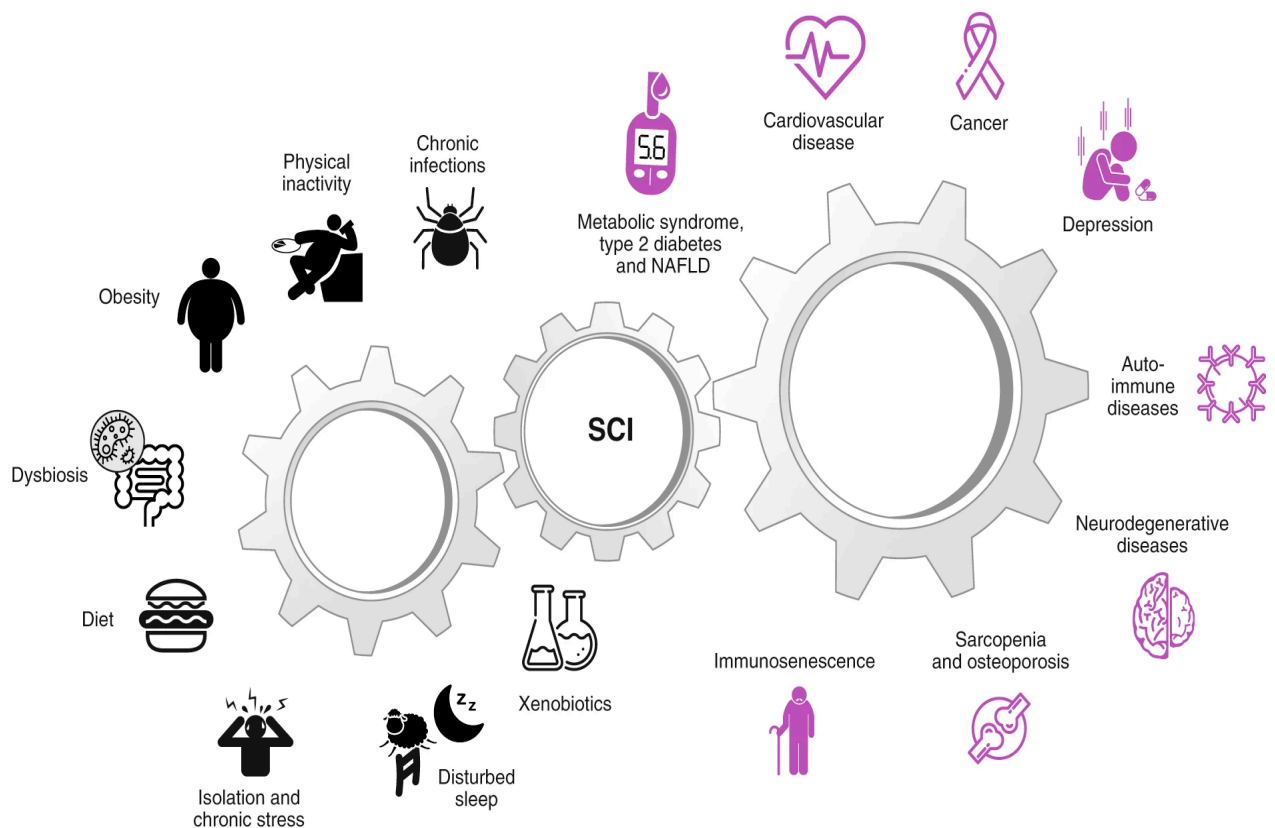


Figure 1-23 Causes of low-grade systemic chronic inflammation (SCI) and their consequences

Developmental origins of chronic inflammation

It has been suggested that the origins of CI can be traced to parental exposures and exposures during early and later childhood. For example, maternal environmental exposures such as infections, dietary exposures, psychological stress during intrauterine life have an effect; also, paternal exposures have an epigenetic effect, the combined effects of which create the potential for inter-generational transmission of risk for CI.

In childhood, factors such as microbial exposure, obesity and psychological stress are known to have an impact on the risk of chronic inflammatory conditions in later life. (Furman, Campisi et al. 2019) With the above risk factors very early in life, other factors already mentioned such as physical inactivity, poor diet, intestinal dysbiosis and psycho-social factors further increase the risk of CI throughout life.

1.3.2 Impact, mechanisms, and consequences of chronic inflammation

It is well known that persistent or non-resolving CI has significant and far-reaching consequences. Perhaps no single phenomenon contributes more to the medical burden in industrialized societies than non-resolving inflammation. (Nathan and Ding 2010) Although non-resolving CI does not primarily cause diseases such as IBD, asthma, chronic obstructive pulmonary disease, rheumatoid arthritis, neurodegenerative disease, multiple sclerosis and cancer, it certainly contributes significantly to their pathogenesis.

Often, acute and chronic inflammation coexist over long periods, implying continual re-initiation and ongoing tissue damage. (Nathan and Ding 2010) This is a complex mechanism, involving pro and anti-inflammatory cells and multiple molecules of the immune system. A schematic

representation of cells and molecules involved is illustrated in **Figure 1-24**, adapted from Calder et al.

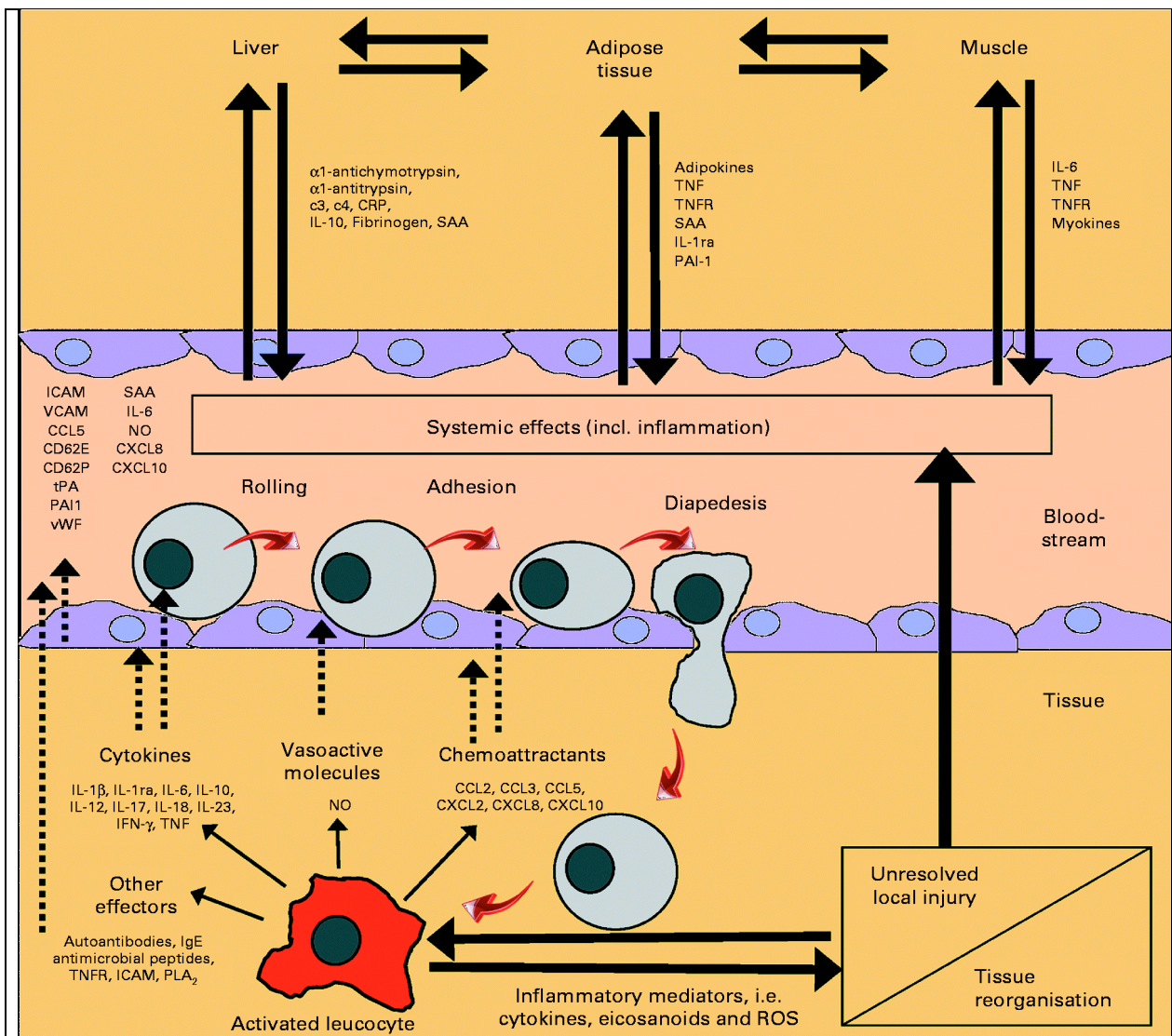


Figure 1-24 An overview of inflammation, cells, cytokines, chemokines and other molecules involved

C, complement; CRP, C-reactive protein; SAA, serum amyloid A; TNFR, TNF receptor; ra, receptor antagonist; PAI, plasminogen activator inhibitor; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; CCL, chemokine (C–C motif) ligand; CD62E, E-selectin endothelial leukocyte adhesion molecule 1; CD62P, P-selectin endothelial leukocyte adhesion molecule 1; CXCL, chemokine (C–X–C motif) ligand; tPA, tissue plasminogen activator; vWF, von Willebrand factor; IFN, interferon; PLA, phospholipase A; ROS, reactive oxygen species.

(Figure adapted with permission from Calder et al)

Non-resolution of CI could be due to many factors but generally due to – a. persistent stimulation
 b. prolonged or excessive response c. subnormal response. This is illustrated in **Figure 1-25**. The end result of CI is usually organ damage and complications.

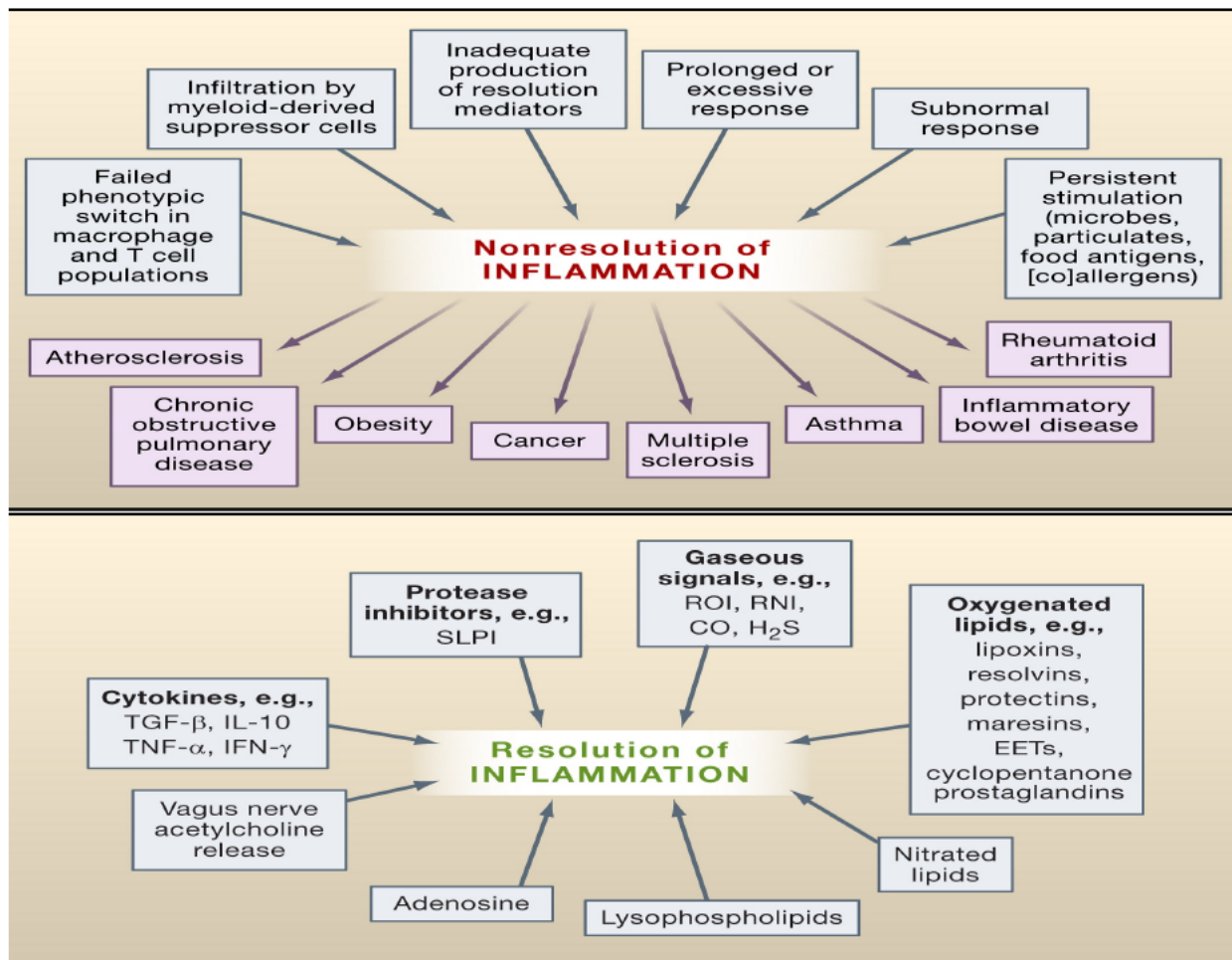


Figure 1-25 Factors contributing to non-resolving inflammation and molecules involved in maintaining balance towards resolution of inflammation

(Figure adapted with permission from Nathan and Ding)

There is a delicate balance maintained within the immune system that helps in tissue repair without causing excessive damage to the host. For post-inflammatory tissue repair to be successful, it is essential that there is coordinated restitution of different cell types and structures,

such as epithelial, mesenchymal cells, extracellular matrix (ECM) and the vasculature. (Nathan and Ding 2010) There are multiple checkpoints that are present within the immune system driven by molecular signals and genes that enable this function.(Nathan 2002) Without appropriate restitution there is resulting atrophy or fibrosis. Over a period of time, these persistent processes destroy the organ involved. Atrophy is often accompanied by expansion of extracellular tissue elements, particularly collagen, resulting in fibrosis. It is also likely that atrophy sometimes promotes fibrosis and fibrosis sometimes promotes atrophy. They can each occur independently too. Fibrosis is a major medical problem and can affect many organ systems including the intestine, where fibrotic strictures occur, often requiring surgery. This will be discussed in greater detail in subsequent sections.

1.3.3 Complications of chronic inflammation in IBD

In the context of IBD, non-resolving CI can cause several serious complications. There are various ways in which they could be classified. Several complications occur as a result of direct intestinal damage. In addition, there are also extra-intestinal complications that occur. A comprehensive list of complications is given in **Table 1-4** classified into extra-intestinal manifestations (EIMs) and extraintestinal complications. (Rothfuss, Stange et al. 2006) EIMs generally tend to be immune-mediated and complications are related to disease or treatment related.

Table 1-4 Extraintestinal manifestations and complications of IBD as per body systems

Extraintestinal manifestations	Extraintestinal complications
Musculoskeletal diseases	
Peripheral Arthritis	Drug-induced osteoporosis and osteonecrosis
Ankylosing spondylitis	Bacterial infection of joints (fistulization, immunosuppression)
Isolated sacroiliitis	Septic arthritis
Metastatic Crohn's disease	
Mucocutaneous diseases	
Erythema nodosum	Anal fissures
Pyoderma gangraenosum	Fistulas
Aphthous stomatitis/oral ulceration	
Psoriasis?	Acrodermatitis enteropathica (Zinc deficiency)
Epidermolysis bullosa acquisita?	Purpura (Vitamin C and K deficiency)
Sweet Syndrome?	Glossitis (Vitamin B or Zinc deficiency)
Erythema multiforme?	Hair loss (protein deficiency)
	Brittle nail (protein deficiency)
	Perlèche (Iron deficiency)
	Candidiasis (Zinc deficiency, immunosuppression)
	Mucositis/stomatitis (methotrexate)
	Drug-induced rash, allergic exanthema
	Moon-face, acne, stretch marks, skin atrophy (steroid treatment)
Ocular diseases	

Extraintestinal manifestations	Extraintestinal complications
Anterior Uveitis	Night blindness Vitamin A deficiency
Conjunctivitis	Kerathopathy Vitamin A deficiency
Iritis	Opportunistic infections (immunosuppressants)
Skleritis/Episcleritis	
Hepatobiliary diseases	
Primary sclerosing cholangitis	Gall stones in Crohn' disease
Granulomatous Crohn's hepatitis	Fatty liver
Autoimmune chronic active hepatitis?	
Biliary cirrhosis?	
Pancreatic diseases	
Acute pancreatitis	Drug-induced pancreatitis (5-ASA, azathioprine)
Chronic pancreatitis, exocrine insufficiency	Biliary pancreatitis in Crohn's disease
	Duodenal involvement in Crohn's disease
Blood and vascular diseases	
Thromboembolic events	Anaemia (Iron-, folate-, vitamin B12-deficiency)
Autoimmune haemolytic anemia?	Thrombocytosis, leucocytosis
Thrombocytopenic purpura (Moscowitz Syndrome)?	Hypercoagulation: venous thrombosis, thromboembolism
Renal diseases	
Tubular proteinuria	Nephrolithiasis (oxalate stones, uric acid stones)

Extraintestinal manifestations	Extraintestinal complications
Glomerulonephritis?	Local affection involving the urethro-genital system
	Acute interstitial nephritis (drug related SASP, 5-ASA)
Interstitial nephritis?	Drug-induced renal insufficiency (5-ASA, SASP, cyclosporine)
	Renal amyloidosis
Bronchopulmonary diseases	
Chronic bronchitis/bronchiolitis/bronchiectasis	Drug-induced hypersensitivity pneumonitis
Acute laryngotracheitis/tracheal stenosis	Drug related pulmonary fibrosis (methotrexate)
Bronchiolitis obliterans organizing pneumonia	Drug-induced pleuritis
Pleuritis/serositis	Opportunistic infections (immunosuppression)
Cardiac diseases	
Pericarditis	Drug-induced pericarditis (5-ASA)
Myocarditis?/perimyocarditis	
Neurological diseases	
Demyelinating diseases including multiple sclerosis	Peripheral neuropathy (Vitamin B12 deficiency)
Optic neuritis, sensorial hearing loss	Drug-induced leucoencephalopathy (natalizumab, infliximab)
Myasthenia gravis?	Drug-induced polyneuropathia (metronidazole)

(Table adapted with permission from Rothfuss et al)

Among the multiple complications listed, acute inflammation in IBD can cause serious life-threatening complications such as fulminant colitis, perforation, abscess formation, sepsis and death. Unchecked or untreated low grade CI has its own complications which can be subacute or long term. By far the most frequently encountered complications encountered in clinical practice are related to nutritional deficiencies. Patients with IBD frequently suffer from anaemia, malnutrition and micronutrient deficiencies (Filippi, Al-Jaouni et al. 2006) This is likely secondary to malabsorption and catabolic state due to low grade CI. This is an important concern for both clinicians and patients. (Prince, Whelan et al. 2011)

Among the many long term complications of CI in IBD, the significant ones encountered in clinical practice are stricturing related to fibrosis (more commonly with CD) and IBD-related colorectal cancer.

1.3.4 Long term complications of chronic inflammation in IBD

1.3.4.1 Fibrosis in IBD

As mentioned earlier, fibrosis is a consequence of CI. In patients with IBD, although it is more commonly seen as a complication of CD, it has been reported in patients with UC too. (Gordon IO 2018)

Mechanisms of fibrosis and stricture formation

CD is characterized by chronic inflammation, ulcerations, and bowel injury followed by recurrent attempts at healing. The gaps caused by multiple recurrent ulcerations are closed by disorganized deposition of ECM. The ECM is formed primarily of collagen subtypes III, V, and I deposited in the

submucosal spaces as well in muscular layers by an increased number of activated fibroblasts and myofibroblasts.(Graham, Diegelmann et al. 1988, Shelley-Fraser, Borley et al. 2012) Fibrosis in CD is due to an exaggerated response characterized by accumulation of collagen-rich ECM, produced by a permanent or transient numerical expansion of mesenchymal cells, including fibroblasts, myofibroblasts and smooth muscle cells.(Rieder, Fiocchi et al. 2017) Fibrosis involves the full thickness of the bowel wall that includes the mucosa, submucosa, muscularis propria and serosa layers resulting in transmural thickening and stiffening with narrowing of the intestinal lumen leading to strictures and obstruction.(Graham, Diegelmann et al. 1988)

The most important recognised driving force behind development of fibrosis is the cytokine transforming growth factor- β 1 (TGF β 1) although other growth factors and cytokines such as interleukin (IL)-4, IL-5, IL-13, platelet-derived growth factor, connective tissue growth factor, basic fibroblast growth factor, insulin-like growth factor, and bone morphogenetic proteins are also involved in the pathophysiology of fibrosis.(Medina, Santos-Martinez et al. 2011) There are several other molecular mediators of fibrosis and most important of these include activins, Connective Tissue Growth Factor (CTGF), Platelet Derived Growth Factor (PDGF), Insulin-like Growth Factor (IGF-1&2), Epidermal Growth Factor (EGF), ET-1, -2, -3, and various cytokines and products of oxidative stress.(Lawrance, Rogler et al. 2017)

1.3.4.2 IBD related Colorectal cancer

Colorectal cancer is one of the most serious complications of IBD. CI is known to be a major risk factor in the pathogenesis of most cancers. The chronic inflammatory state in the GI tract due to IBD is one of biggest risk factors for development of IBD related colorectal cancer (IBD-80

CRC).(Majumder, Shivaji et al. 2022) Although only 1-2% of IBD patients develop IBD-CRC, it contributes to about 15% of IBD related mortality.(Leong and Koo 2009) As per current long term epidemiological data, the risk of colorectal cancer (CRC) in IBD patients can be as high as 18% after 30 years of diagnosis, (Eaden, Abrams et al. 2001) although this may have improved over time. The role of CD in the development of CRC is debatable and considered modest in comparison to UC. Canavan et al in their meta-analysis estimated cumulative risk of 2.9% at 10 years, 5.6% at 20 years and 8.3% at 30 years in patients with CD.(Canavan, Abrams et al. 2006) The severity of inflammation is a significant risk factor that increases the risk of IBD-CRC. Inflammation related oxidative stress leading to genomic instability is considered the main trigger. Cellular and molecular studies on colonic tissue in IBD-CRC have found that the sequence of development of carcinogenesis is different from that observed in sporadic cancer in the non-inflamed colon. IBD-associated carcinogenesis follows an 'inflammation-dysplasia-carcinoma' sequence instead of the 'adenoma-carcinoma' sequence seen in sporadic CRC.(Itzkowitz and Yio 2004) As a result, they may accelerate much faster to carcinoma compared to sporadic cancers. There are multiple factors that contribute to the risk of IBD-CRC (**Figure 1-26**) but persistent inflammation is perhaps the most important.

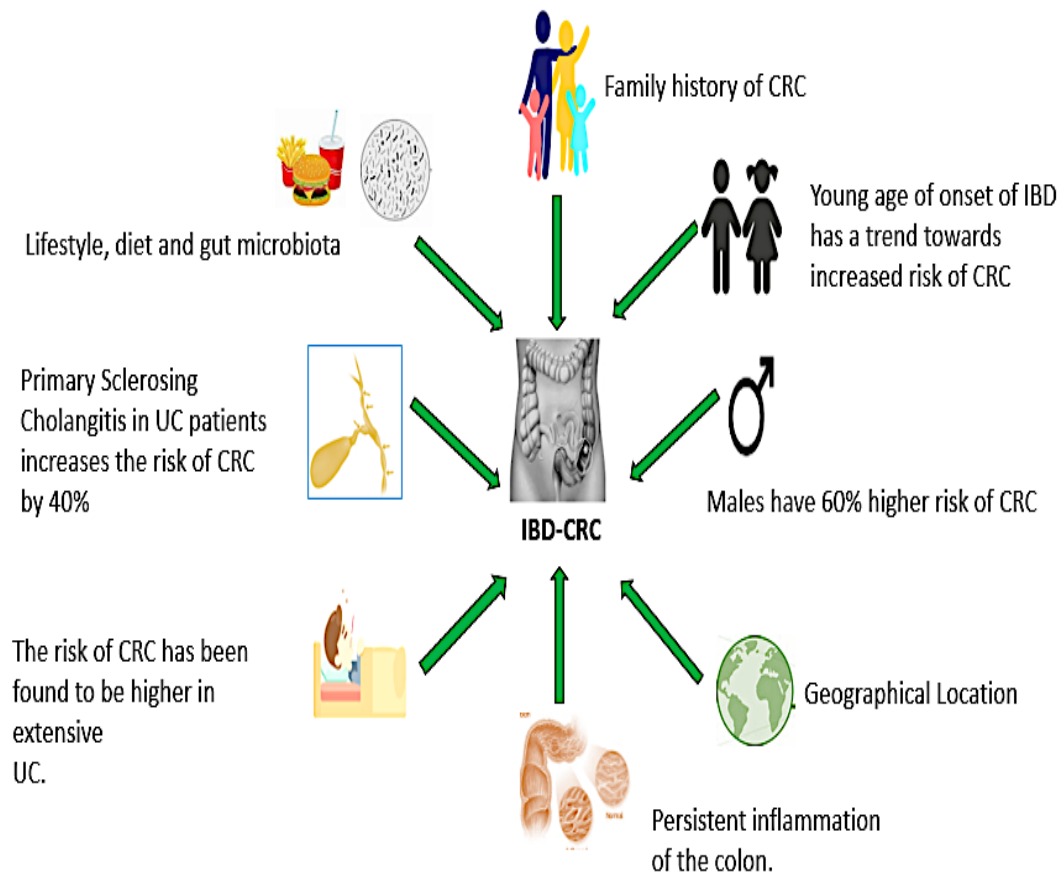


Figure 1-26 Risk factors for development of IBD related Colorectal cancer

(Figure adapted with permission from Majumder et al)

1.3.5 Biomarkers of chronic inflammation

Despite better understanding of the process, there are currently no reliable biomarkers to identify or prognosticate chronic inflammation. This is an area of ongoing research. The conventional biomarkers lack specificity and are unable to predict disease course, morbidity, or mortality reliably. Existing biomarkers, which include CRP, IL-1 β , IL-6 and TNF- α , to some extent are useful to demonstrate that inflammatory activity is related to disease and mortality risk. However, they provide limited mechanistic information considering the complexity of inflammatory response. Also, they do not address anti-inflammatory regulatory pathways that may also be relevant for influencing inflammation-related disease risk. (Furman, Campisi et al. 2019) On the other extreme are biomarkers of mucosal healing which could reliably detect healing of the intestine thereby acting as a negative marker for CI. (Nardone, Shivaji et al. 2020)

At present Faecal calprotectin (FCP) and CRP are perhaps the most commonly used biomarkers for monitoring purposes in IBD in clinical practice. FCP has established itself as a cheap and reliable biomarker in IBD. Although they have been reliable over time, they are not specific and can vary with many factors.

To overcome these limitations several research groups have attempted to identify an ideal biomarker in IBD. One such soluble biomarker is the Monitr test (Prometheus, San Diego, CA), a serological test developed to unify many potential promising biomarkers established or explored in CD. (Nardone, Shivaji et al. 2020) It consists of 13 biomarkers and in a study reported by Sandborn et al, the test predicted mucosal healing in CD patients with an accuracy of 95%, 90%, and 87% for ileal, ileocolonic, and colonic disease, respectively. (Sandborn, Abreu et al. 2018) The availability of these biomarkers may reduce the proportion of patients with sustained CI.

More recently, with availability of newer technology, a multi-omics approach has been applied to examine links between CI and disease risk itself. In one study, the investigators longitudinally followed 135 adults and conducted deep molecular profiling of participants' whole-blood gene expression, immune proteins, and cell subset frequencies e.g., - CD8⁺ T cell subsets, monocytes, natural killer (NK) cells, B cells and CD4⁺ T cell subsets. This deep analysis allowed researchers to construct a trajectory of immune aging (IMM-AGE) that described individuals' immune functioning better than their chronological age.(Alpert, Pickman et al. 2019, Furman, Campisi et al. 2019) This new metric was able to accurately predict all-cause mortality, establishing its potential future use for identifying at-risk patients. Similar approaches may become available for specific conditions too. There are several techniques and principles that can be applied to develop biomarkers in the future. Some examples are- cell surface markers, cluster of biomarkers, microRNA, stress markers microbiota and so on. (Calder, Ahluwalia et al. 2013)

It is likely that the most widely used and accepted tests by patients in clinical care, will be non-invasive or soluble biomarkers that can be detected using blood. A reliable biomarker could guide clinical management, decrease number of invasive procedures, improve early intervention, and recognise flares early. They are also more likely to be accepted as point-of-care or home tests. (Nardone, Shivaji et al. 2020) With more advanced technology, we will possibly see the emergence of many new biomarkers and panels that could detect CI and MH. A part of our work aimed at investigating biomarkers that could serve as reliable indicators of CI and MH , in addition to looking for therapeutic targets.

1.3.6 Rationale for studies discussed in thesis

There is little doubt that sustained chronic inflammation results in many complications in the long term. Equally, there is growing evidence clinically that aggressive management of inflammation reduces complications, rates of surgery and hospitalisation in CD.(Mandel MD1 2014) Mucosal healing and histological healing are now accepted as targets of therapy that are known to reduce complications and disease related adverse events in the future. However, the deep histological changes or molecular mechanisms to explain lower complications have seldom been reported. There is a gap in knowledge that requires more research and evidence. Deeper analysis of histology in select group of patients who have significant complications such as strictures alone may provide a better understanding of the microenvironment within the bowel wall. Studying molecular markers in diseased segment of the intestine using advanced techniques may reveal some newer targets of therapy.

One of the complications of CI that is common and quite challenging to manage is fibrosis or stricturing in CD. Fibrosis has been shown to be reversible with certain pharmacotherapies in other organs systems, (Rieder, Fiocchi et al. 2017) but there are no effective anti-fibrotic therapies known yet which could manage intestinal fibrosis. This is an unmet need. Bettenworth et al report in one study that there may be a degree of reversibility to strictures, but this will need further investigation. They suggest that multiple distinct signalling cascades contribute to fibrosis, and these could be potential therapeutic targets. The selective targeting of cell types could be one approach, potentially stimulating those which may have an anti-fibrotic effect.(Bettenworth and Rieder 2016)

The studies discussed in the thesis were designed to better understand CI in IBD and explore biomarkers of CI and its fallouts. A deeper understanding of molecular aspects may provide novel targets of therapy for treatment of IBD and complications of IBD such as fibrosis and possibly IBD related CRC.

The broad collective aims of the studies/projects discussed were mainly to -

- Understand deep histological aspects of CD related strictures
- Understand the impact of chronic persistent inflammation (sub-optimal control of inflammation) on patients with IBD, at different stages of their disease journey (e.g.- post-surgical, delayed first-diagnosis, patients already on biologic therapies) with clinical outcomes
- Study molecular signals, biomarkers and novel pathways involved in chronic inflammation and fibrosis
- Study immuno-histochemistry for novel cytokines in CD strictures
- Study and identify novel predictive biomarkers of CI and mucosal healing

We planned the studies in three phases-

- **Phase 1** - retrospective analysis of histology samples from patient who have undergone surgery for CD
- **Phase 2** – study clinical outcomes of delayed diagnosis of IBD and biologic discontinuation of first-use biologic in IBD
- **Phase 3** – design and implementation of a prospective study (TRAFIC Study), designed to study molecular signals of CI in IBD, biomarkers and pathways, with a focus on fibrosis

This is illustrated in the Study flow chart below. **(Figure 1-27)**

Study flowchart

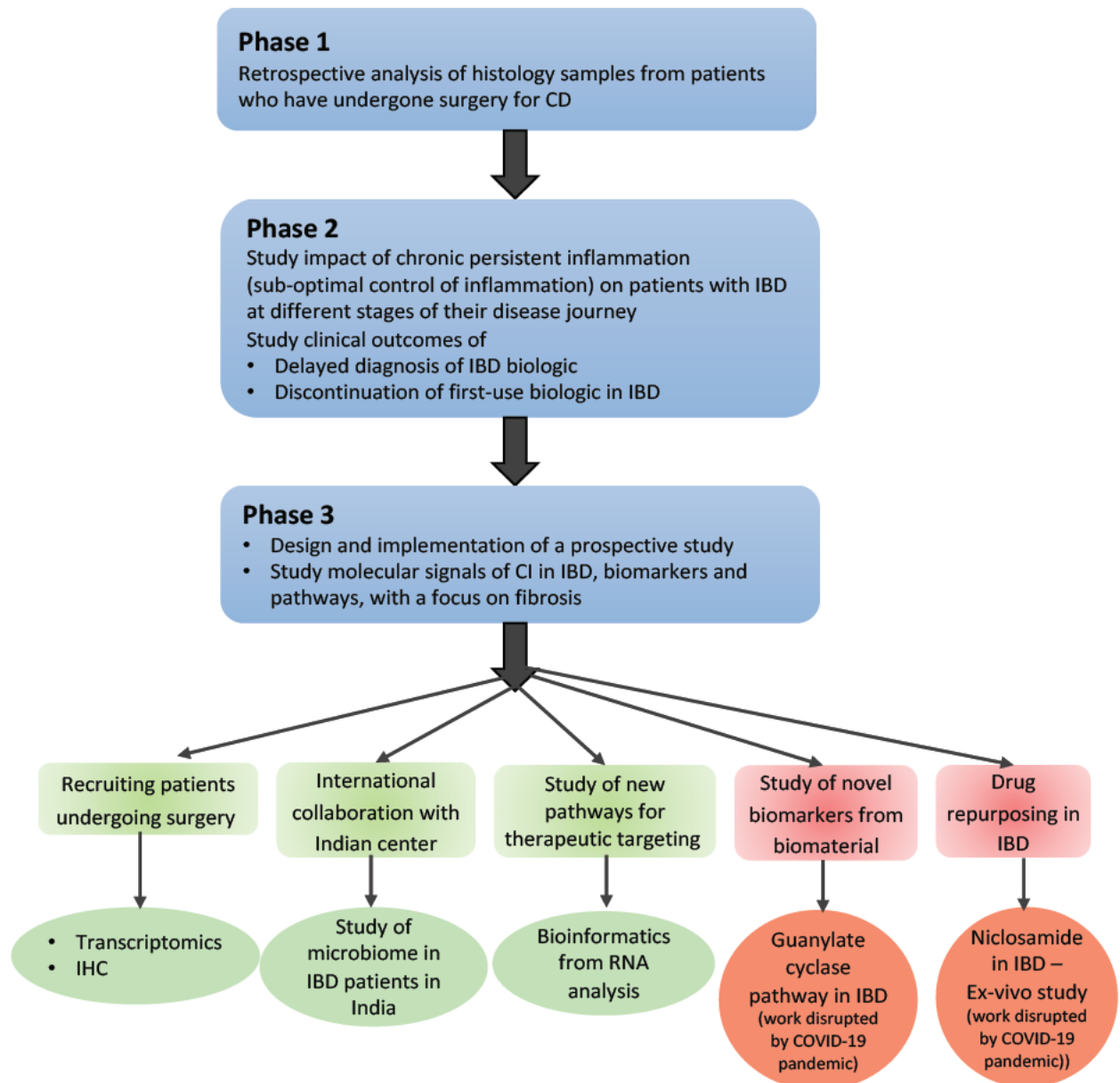


Figure 1-27 Flowchart of projects done in phases

This flowchart given details of each phase and projects undertaken in Phase 1,2 and 3.

Phase 3 study has multiple stems. This study remains open for recruitment both locally and internationally.

■ indicates ongoing work or completed work.

■ indicates studies that had to be stopped mid-way due to disruption during the COVID-19 pandemic.

2 CHAPTER 2: RETROSPECTIVE STUDY ON HISTOPATHOLOGICAL CHANGES SEEN IN CROHN'S DISEASE RELATED FIBROTIC STRICTURES

Parts of this chapter have been published as-

Shivaji UN, Evans M, Critchlow T, et al. Chronic inflammation and other changes are significant components of clinically fibrotic strictures in Crohn's disease: a histological study of resected strictures clinically characterized as noninflamed [published online ahead of print, 2020 Jul 3]. *Eur J Gastroenterol Hepatol*. 2020 (Shivaji, Evans et al. 2020)

doi:10.1097/MEG.0000000000001796

Overview of chapter

This chapter includes discussion of a detailed study undertaken to investigate histological changes seen in fibrotic strictures of patients with Crohn's Disease. A comprehensive histological scoring system developed specifically for intestinal strictures was applied. In this study, all patients who were deemed to have non-inflamed strictures prior to surgery were included. The results of the study gave us better understanding of the changes occurring within the bowel wall of patients with CD. Results from this study also helped us design a prospective study to investigate the microenvironment of intestinal strictures which could inform long-term treatment strategies and provide newer targets of therapy.

Chronic inflammation is undoubtedly the significant underlying issue in most autoimmune conditions, and this is the case in IBD also. The study of acute and chronic inflammation is best achieved by investigating the tissue involved in the condition. A retrospective study was undertaken to understand this better. We began by selecting and examining histological samples available from patients who had undergone intestinal resection for CD related complications such as uncontrolled inflammation, strictures or other adverse abdominal features as indications. This deep histological assessment would give us more insights into components of the intestinal wall which has endured the effects of chronic inflammation at the tissue level.

This chapter presents the results of the retrospective study which guided further work.

2.1 Background and aims of study

A significant proportion of patients who have CD undergo surgery at some point in their disease journey.(Baumgart and Sandborn 2012) In most cases it is due to poor or suboptimal response to medical therapy and a consequence of poorly controlled chronic inflammation. A small proportion of patients may present acutely with a complication such as perforation of the intestine or an abscess, before the diagnosis is known. The common underlying cause of most complications is uncontrolled inflammation, and this is worthy of further inspection. As part of our long-term research roadmap to understand more about molecular signals and biomarkers in IBD, we first wanted to study the composition of strictures & inflamed intestinal tissue better. This knowledge would no doubt be useful in managing patients in the future more effectively and guide our work.

There are multiple phenotypes in CD. Although the most common presentation of CD is inflammatory, approximately 50% of patients develop stricturing (Montreal B2) or penetrating (B3) complications in the first 10 years after diagnosis.(Louis, Collard et al. 2001, Cosnes, Cattan et al. 2002) Strictures are formed as a result of intestinal fibrosis and can occur at any time and involve any intestinal segment. The commonest sites for strictures in CD are the terminal ileum and ileo-colonic region.(Peyrin-Biroulet, Loftus et al. 2010) These strictures frequently require surgery if they begin to cause intestinal obstruction. The overall risk of intestinal surgery in IBD patients has decreased over the past six decades but despite this decrease, the risk remains high (16.3%, 33.3%, and 46.6% at 1, 5, and 10 years, respectively, after diagnosis of CD)(Frolkis, Dykeman et al. 2013)

In active CD there is ongoing bowel damage, followed by recurrent attempts at healing. The damage caused by multiple recurrent ulcerations is repaired by disorganized deposition of extracellular matrix (ECM). The ECM is formed primarily of collagen subtypes I, III, and V deposited in the submucosal spaces as well as in muscular layers by an increased number of activated fibroblasts & myofibroblasts.(Graham, Diegelmann et al. 1988, Shelley-Fraser, Borley et al. 2012) Fibrosis in CD is caused due to an exaggerated response characterized by accumulation of collagen-rich ECM, produced by mesenchymal cells, including fibroblasts, myofibroblasts and smooth muscle cells(Rieder, Fiocchi et al. 2017) and driven by cytokines & growth factors, most notably transforming growth factor- β 1 (TGF β 1).(Medina, Santos-Martinez et al. 2011) Due to transmural nature of inflammation in CD, fibrosis involves the full thickness of the bowel wall resulting in transmural thickening, stiffening and narrowing of the intestinal lumen, leading to strictures and obstruction.(Shelley-Fraser, Borley et al. 2012). More recently, there has been growing evidence and interest in the role of fibroblasts that have been shown to promote inflammation within the microenvironment of the tissue resulting in maintaining persistent inflammatory state.(Buckley 2011)

This study was conceptualised with the above relevant information in mind. The purpose was to interrogate the changes seen within the resected segment of the intestine of patients with CD. Patients with CD were selected for this study as they are more likely to have strictures in the intestine, a well-known complication of chronic inflammation.

In routine clinical care, the usual clinical assessments and investigations undertaken prior to planning surgery for patients generally include nutritional assessments, routine blood tests and cross-sectional imaging. Blood tests help assess degree of inflammation; cross sectional imaging techniques that include Computed tomography (CT) and/or Magnetic Resonance Enterography

(MRE) are used to determine the extent of disease, location of strictures and length & character of strictures. Imaging has become integral to disease activity assessment and has been used to guide management for some years now.

The aims of this study were-

- to examine detailed histological characteristics of strictures in CD in post-surgical resected intestinal segments.
- to examine radiological features of the intestine prior to surgery, and analyse if there are any adverse features that could predict poor outcomes
- to correlate radiological features with histology and study the short term and long-term outcomes of disease

2.2 Patients and Methods

All patients undergoing surgery for CD secondary to symptomatic ‘fibrotic’ stricturing disease between 2012 and 2017 were identified using the histopathology database at University Hospitals Birmingham NHS Foundation Trust, UK. The diagnosis of a stricture was made based on a combination of clinical symptoms, endoscopic and radiological findings (CT and/or MRE). Inflammation was assessed based on MRE appearance, colonoscopy & biopsies, where available, and C-Reactive Protein (CRP). All resections were undertaken based on cross-sectional imaging showing narrowing with pre-stenotic dilatation associated with clinical episodes of sub-acute intestinal obstruction.

48 patients were included and there was proportional distribution between sexes. (M=25) The median age was 45 years (range 21-72 years) with 41 Caucasian, 4 Asian and 3 Afro-Caribbean in ethnicity. The details of demographics, disease classification and medications are in **Table 2-1**.

The median duration of disease from diagnosis was 7 years (range 0.25-39 years). Majority of patients (nearly two-thirds) had ileo-colonic distribution (L3) of disease, and all had stricturing disease (B2). All patients were followed up for clinical outcomes (recurrence, complications, change of therapy, surgery) until December 2018. As the patient inclusion in the study spanned many number of years, they had varying lengths of follow-up (median range of follow up was 45 months; range 24-60 months) depending on their year of surgery. The indication for surgical resection was fibrotic stricture/s based on cross-sectional imaging, disease course, response to treatment, and finally decided at a multidisciplinary meeting involving colorectal surgeons, IBD gastroenterologists, radiologists and histopathologists.

Table 2-1 Patient demographics and characteristics

Age & gender	
Median age	45 years (range 21 to 72 years)
Gender	Male=25; Female=23
Disease duration from diagnosis	
<10 years	24
>10 years	24
Median duration	7 years (0.25 - 39 years)
Medications at the time of resection	
Mesalazine	4
Corticosteroids	19
Immunomodulators	16
Biologics	4

All clinical data including details of phenotypic data were recorded from Electronic Medical Records (EMR), and the resection specimens were reviewed. Macroscopic data were extracted from the histopathology reports. Although only 20 patients were on a combination of immunosuppressives and/or biologics at the time of resection, all patients had been declared steroids failures and reached ceiling of medical management.

Methodology of histological grading/scoring

Two pathologists who were blind to any clinical information, applied the Calgary Histological Grading system [developed by us, Chen W et al(Chen, Lu et al. 2017)] independently, in a comparable way, collecting data electronically, and agreeing on the final scores. This scoring system is a very comprehensive one and involved detailed examination of each layer for different components. A systematic review by De Voogd examined various scoring systems available for deep histological scoring in CD. The scoring system by Chen et al had a high score for quality based on their assessment. (De Voogd, Mookhoek et al. 2020)

To apply this grading/scoring system, the haematoxylin and eosin-stained slides that were deemed to be most representative of the stricture/fibrotic segment were used and as these were surgical specimen, full thickness sections were selected. For each layer of the bowel wall, four 100x microscopic fields were scored and the average score was obtained on each histopathological component. The different components scored in appropriate layers of the bowel were -

- active inflammation
- adipocyte (fat) hyperplasia
- chronic inflammation
- fibrosis
- hypertrophy in of muscularis propria

- neuronal hypertrophy
- smooth muscle hyperplasia in submucosa
- space volume expansion

The definitions used to identify each component are given in **Table 2-2**. The histological features in each layer of bowel wall were scored and further adjusted for comparison by calculating its percentage [%] of the possible maximum total score [PMTS] for each category in each layer: adjusted score [%] = [actual total score/PMTS] x 100. The global score of a component was calculated as the sum of scores in each layer of the bowel wall. Data was analysed using the %PMTS (possible maximum total score) recorded by the pathologists and all data were included in our final analysis. The details of the semi-quantitative scoring scheme are in **Table 2-3**.(Chen, Lu et al. 2017)

Table 2-2 Details of components scored and respective definitions in this study

Components scored	Definition used for each component in this study
Active inflammation	Superficial ulceration, fissuring, cryptitis, crypt abscess and neutrophil infiltration in lamina propria.
Adipocyte (fat) hyperplasia	Marked presence of adipocytes
Chronic inflammation	Lamina propria mononuclear cell density, eosinophil infiltration, lymphoid follicles/aggregates and crypt architecture alteration
Fibrosis	Fibroblast proliferation mixed with collagen and other matrix deposition
Hypertrophy in muscularis propria	Marked thickening of smooth muscle layers as compared to normal bowel
Neuronal hypertrophy	Marked thickening of neuronal components
Smooth muscle hyperplasia in submucosa	Marked smooth muscle bundles dissection

Space volume expansion	Volume expansion of each layer was also graded by comparing the thickness of the layer with that of the relatively normal regions of the same intestinal tract
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Table 2-3 Histological grading/scoring scheme of Crohn's fibrostenosis

	Normal [score 0]	Mild [score 1]	Moderate [score 2]	Severe [score 3]
1. Mucosa				
Active inflammation				
Superficial ulcer [affected surface %]	None	< 33% or erosion	> 33% and < 66%	> 66%
Fissuring ulcer	None	Focal	Multifocal	Extensive
Cryptitis	None	< 5%	> 5% and < 50%	> 50%
Crypt abscess	None	Focal	Multifocal	Extensive
Lamina propria PMNs infiltration	None	Rare	Patchy	Extensive
Chronic inflammation				
Lamina propria lymphoplasmacytic cellularity	Normal	Mild increase	Moderate increase	Severe increase
Lamina propria eosinophilic infiltration	Normal limit	Mild	Moderate	Severe
Lymphoid follicles/aggregates	None	< 1 PLF	> 1 and < 3 PLF	> 4 PLF
Crypt architecture alteration	None	< 33%	> 33% and < 66%	> 66%
Fibrosis [extent]	None	< 33%	> 33% and < 66%	> 66%
Muscular hyperplasia				
Lamina propria smooth muscle bundle dissection	None	< 33%	> 33% and < 66%	> 66%
Muscularis mucosae thickening [x normal mucosa]	Normal	< 2x	> 2x and < 3x	> 3x
Space volume expansion	None	< 2x	> 2x and < 3x	> 3x
2. Submucosa				
Active inflammation				
Involved by fissuring ulcer	None	Focal	Multifocal	Extensive
Abscess	None	Focal	Multifocal	Extensive
PMNs infiltration [in non-ulcer/abscess area]	None	Rare	Patchy	Extensive
Chronic inflammation				
Lymphoplasmacytic infiltration	None	Rare	Patchy	Extensive
Eosinophilic infiltration	None	Mild	Moderate	Severe
Lymphoid follicles/aggregates	None	< 1 PLF	> 1 and < 3 PLF	> 4 PLF

Fibrosis [extent]	None	< 33%	> 33% and < 66%	> 66%
Muscular hyperplasia				
Smooth muscle bundle proliferation [extent]	None	< 33%	> 33% and < 66%	> 66%
Muscularis mucosae downward dissection	None	< 33%	> 33% and < 66%	> 66%
Adipocyte proliferation	None	< 33%	> 33% and < 66%	> 66%
Neuronal hypertrophy	None	< 1 PLF	> 1 and < 3 PLF	> 4 PLF
Space volume expansion	None	< 2x	> 2x and < 3x	> 3x
3. Muscularis propria				
Active inflammation				
Involved by fissuring ulcer	None	Focal	Multifocal	Extensive
Abscess	None	Focal	Multifocal	Extensive
PMNs infiltration [in non-ulcer/abscess area]	None	Rare	Patchy	Extensive
Chronic inflammation				
Lymphoplasmacytic infiltration	None	Rare	Patchy	Extensive
Eosinophilic infiltration	None	Mild	Moderate	Severe
Lymphoid follicles/aggregates	None	< 1 PLF	> 1 and < 3 PLF	> 4 PLF
Fibrosis [extent]	None	< 33%	> 33% and < 66%	> 66%
Muscular hypertrophy				
Longitudinal layer thickening [x normal thickness]	None	< 2x	> 2x and < 3x	> 3x
Circular layer thickening [x normal thickness]	None	< 2x	> 2x and < 3x	> 3x
Neuronal hypertrophy	None	< 1 PLF	> 1 and < 3 PLF	> 4 PLF
Adipocyte proliferation	None	Focal	Multifocal	Extensive
Space volume expansion	None	< 2x	> 2x and < 3x	> 3x
4. Subserosal adventitia				
Active inflammation				
Involved by fissure	None	Focal	Multifocal	Extensive
Abscess	None	Focal	Multifocal	Extensive
PMNs infiltration [in non-fissuring/abscess area]	None	Rare	Patchy	Extensive
Chronic inflammation				
Lymphoplasmacytic infiltration	None	Rare	Patchy	Extensive
Eosinophilic infiltration	None	Mild	Moderate	Severe
Lymphoid follicles/aggregates	None	< 1 PLF	> 1 and < 3 PLF	> 4 PLF
Fibrosis [extent]	None	< 33%	> 33% and < 66%	> 66%
Muscular hyperplasia	None	< 2x	> 2x and < 3x	> 3x

Neuronal hypertrophy	None	< 1 PLF	> 1 and < 3 PLF	> 4 PLF
Space volume expansion	None	< 2x	> 2x and < 3x	> 3x

PMN, polymorphonuclear cells; PLF, per low power field (40 x). Scoring scheme from the original scoring system by Chen et al.

Statistical analysis

All data collected was analysed using IBM SPSS (Statistical Software for Social Sciences) for Windows, Version 24.0 (Armonk, NY: IBM Corp). Correlation between two variables in the histopathology scoring was determined using the Pearson correlation coefficient (r) as a strength of the association between two variables, and a p value of <0.05 was considered statistically significant.

Ethical considerations

This study was approved by the hospital ethical review board. All patients had signed informed consent for their tissue to be used for research purposes and the process was in accordance with the 1975 Declaration of Helsinki.

2.3 Results

The quantitative analysis for the entire cohort is represented in **Figure 2-1**. The histological grading obtained using the scoring system revealed a variable predominant component in each layer of the bowel wall. **(Figure 2-1)** The different components in each layer and a brief narrative of individual scores are described below.

- A. Active/acute inflammation
- B. Chronic inflammation

- C. Fibrosis
- D. Volume expansion
- E. Muscular hyperplasia/ hypertrophy
- F. Neuronal hypertrophy
- G. Adipose hyperplasia

The details of the numerical scores (mean $\pm$ SEM) for each component in the bowel wall are as listed in **Table 2-4**

A. Active/acute inflammation

The score for active inflammation was highest in the mucosa but overall, active inflammation scored low on the global score of %PMTS in the whole bowel wall. It was not a significant component in the sub-mucosa, muscularis propria or sub-serosa. The scoring of this component in individual layers as well as the global score is represented in **Figures 2-1 and 2-2**

B. Chronic inflammation

Chronic inflammation (CI) was the most important and consistent finding across all layers of the bowel wall, with a global score of about 42 (%PMTS) (**Figure 2-2**). The score for CI was highest in the mucosa and the second most important component after fibrosis in the sub-mucosa and sub-serosa. Although CI was seen in the muscularis propria, other components also appeared to be more dominant. CI had a positive correlation with fibrosis (FS) and muscularis propria hyperplasia (MPH) and was statistically significant.

C. Fibrosis

Fibrosis was the third most prominent feature in the mucosa but was the most prominent in the sub-mucosa and sub-serosa. The overall score for fibrosis was highest (global score of %PMTS) in the whole bowel wall, with a numerical score of about 48. This was to be expected given the

cohort of patients included in this study. However, an interesting finding was the association of this component with CI. It is seen to be closely related to chronic inflammation in the sub-mucosa, muscularis propria and sub-serosa layers.

D. Muscular hyperplasia / hypertrophy

Muscular hyperplasia/hypertrophy or muscularis propria hyperplasia (MPH) was the third most prominent component overall. However, it was a consistent feature seen in all layers of the bowel wall with an overall numerical score of 35. MPH was more prominent in the mucosa with reducing prominence in deeper layers of the bowel wall. The correlation of MPH and CI was statistically significant (p-value 0.003; $r=0.422$). This is represented in **Figure 2-5**

E. Volume expansion

Volume expansion (VE) which was graded by comparing thickness of the layer with relatively normal regions of the same intestinal tract at the margins was the next prominent component overall, after MPH. VE was the most prominent component in the muscularis propria, also noted in the sub-mucosa and sub-serosa.

F. Neuronal hypertrophy

Neuronal hypertrophy contributed towards the thickening of bowel wall within the submucosal layer.

G. Adipose hyperplasia

Adipose hyperplasia was noted only in two layers, contributing to thickening more within the sub-mucosa than in the muscularis propria.

Table 2-4 Results based on numerical histological scores (%PMTS)

	Mean	SEM
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1. Mucosa		
Active inflammation	22.92	2.37
Chronic inflammation	52.95	2.56
Fibrosis	40.28	5.15
Muscular hyperplasia	46.88	3.32
Volume expansion	24.31	3.81
2. Submucosa		
Active inflammation	12.50	2.60
Chronic inflammation	48.38	2.86
Fibrosis	70.83	4.40
Muscular hyperplasia	45.14	3.99
Adipocyte proliferation	33.33	4.09
Neuronal hypertrophy	31.94	5.87
Volume expansion	39.58	4.05
3. Muscularis propria		
Active inflammation	6.02	2.04
Chronic inflammation	28.24	2.96
Fibrosis	31.25	3.77
Muscular hyperplasia	30.90	2.86
Neuronal hypertrophy	22.92	4.46
Adipocyte proliferation	4.86	1.72
Volume expansion	38.89	3.34
4. Subserosa		
Active inflammation	4.02	1.19
Chronic inflammation	38.06	2.94
Fibrosis	53.19	5.13
Muscular hyperplasia	21.99	4.09
Neuronal hypertrophy	26.24	5.06
Volume expansion	36.88	4.45

SEM, standard error of the mean; PMTS, possible maximum total score

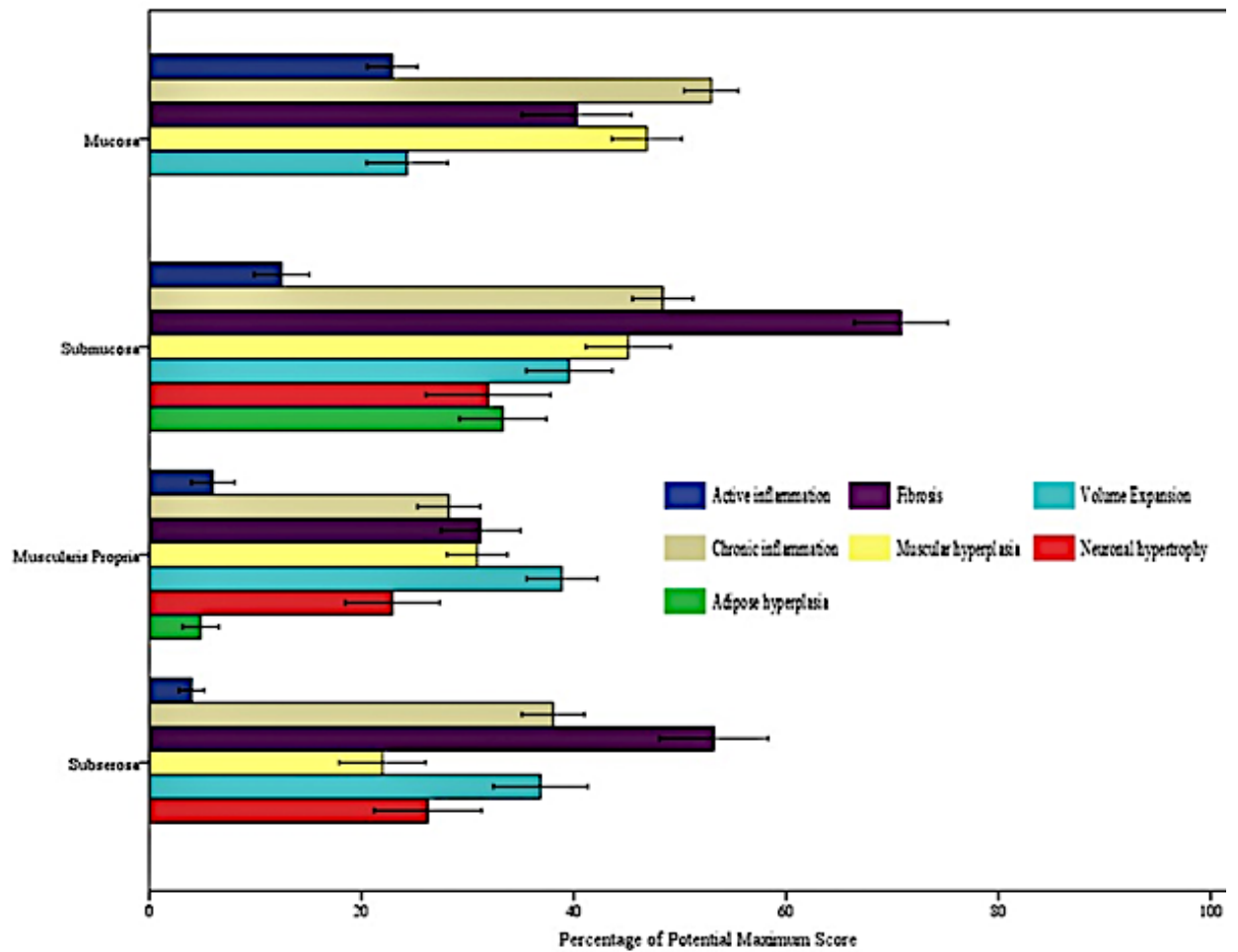


Figure 2-1 Histological scores [%PMTS] in each bowel layer

(Figure adapted with permission from Shivaji et al)

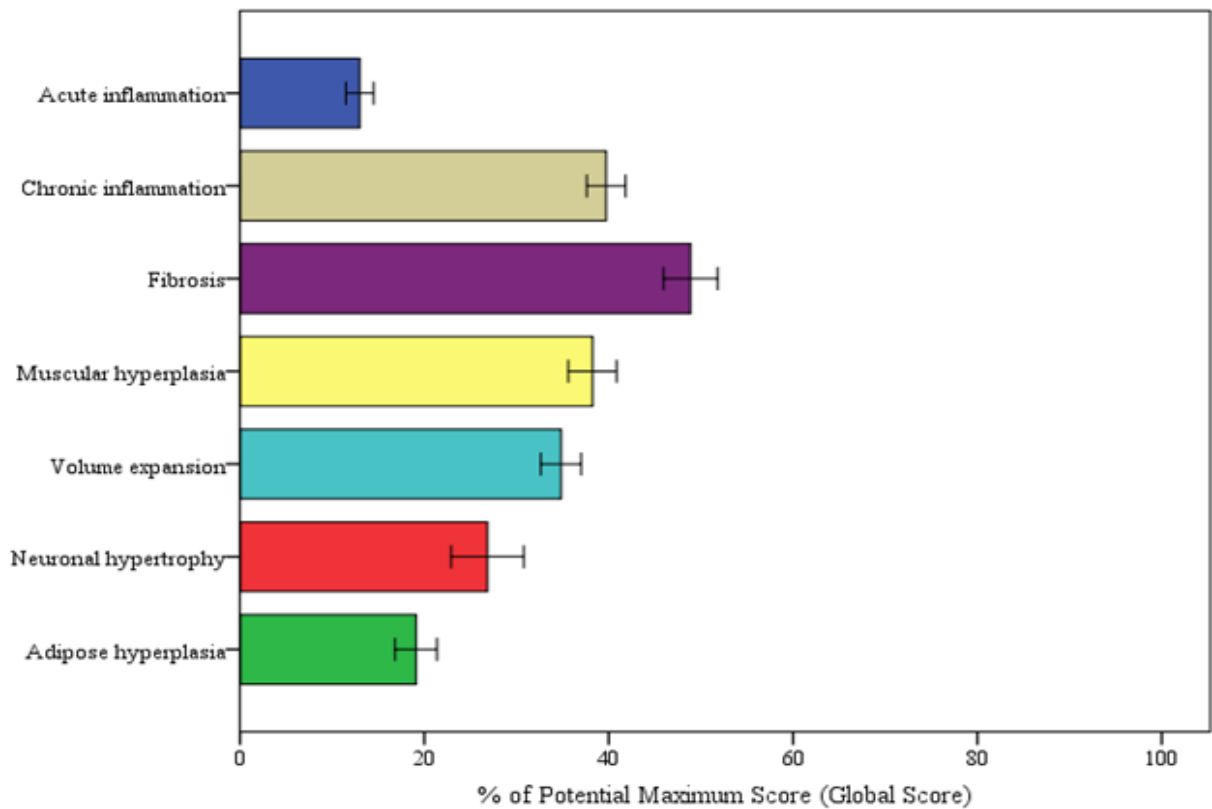


Figure 2-2 Global score in the whole bowel wall

(Figure adapted with permission from Shivaji et al)

2.4 Correlation between different components

Further analysis of the correlation of all components with each other was carried out, with specific focus on inflammation and fibrosis as the highest scoring components. (As per global %PMTS).

These are demonstrated in **Table 2-5**.

There was statistically significant positive correlation between chronic inflammation and fibrosis (**Fig 2-3**) and muscular hyperplasia and fibrosis (**Fig 2-4**). Further details of correlation of each of the components by individual layers with remaining components (using numerical scores), are given in **Table 6-1**. (this is included in Appendix 1)

Table 2-5 Global analysis of correlation between different components

Correlation between	Pearson correlation- r	p-value
AI <-> CI	0.378	0.008
CI <-> FS	0.339	0.018
CI <-> MPH	0.422	0.003
CI <-> VE	0.565	<0.001
FS <-> MPH	0.648	<0.001
FS <-> NH	0.375	0.009
FS <-> VE	0.528	<0.001
MPH <-> NH	0.493	<0.001
MPH <-> VE	0.778	<0.001
NH <->VE	0.452	0.001

AI-acute inflammation, CI- chronic inflammation, FS-fibrosis, MPH-muscular hyperplasia, NH-neuronal hypertrophy, VE-volume expansion

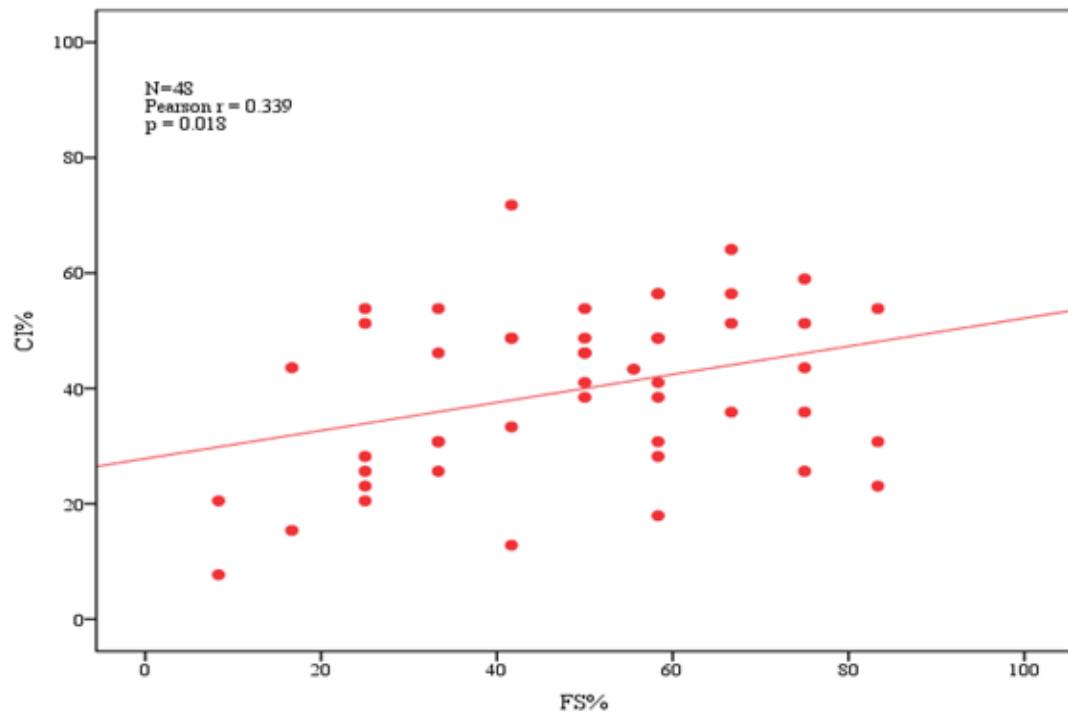


Figure 2-3 Correlation between chronic inflammation (CI) and fibrosis (FS) (by global %PMTS)

(Figure adapted with permission from Shivaji et al)

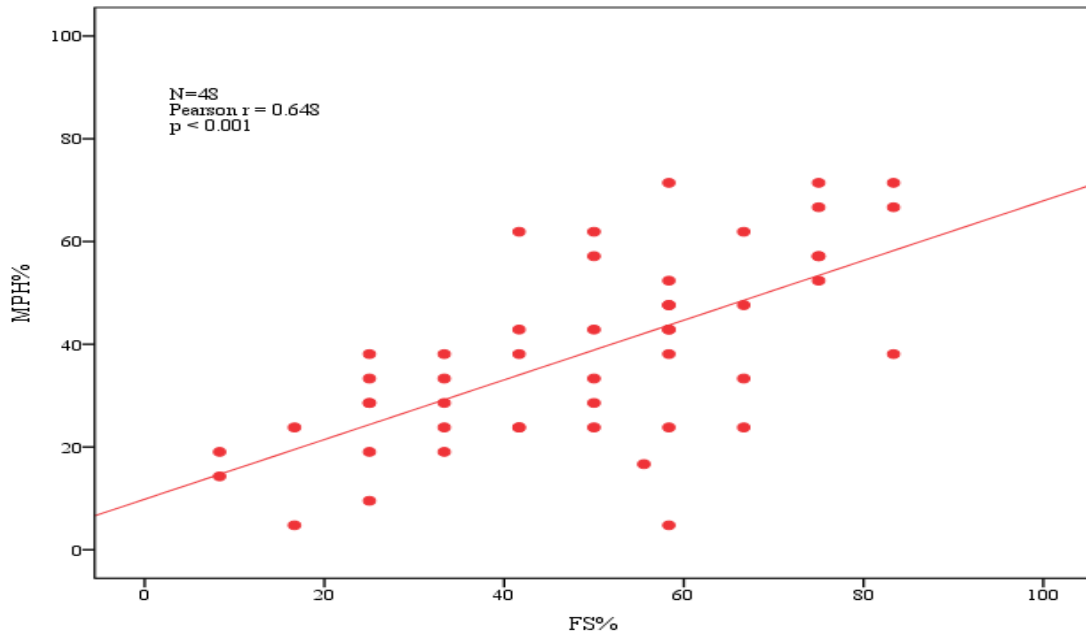


Figure 2-4 Correlation between muscular hyperplasia (MPH) and fibrosis (FS) (by global %PMTS)

(Figure adapted with permission from Shivaji et al)

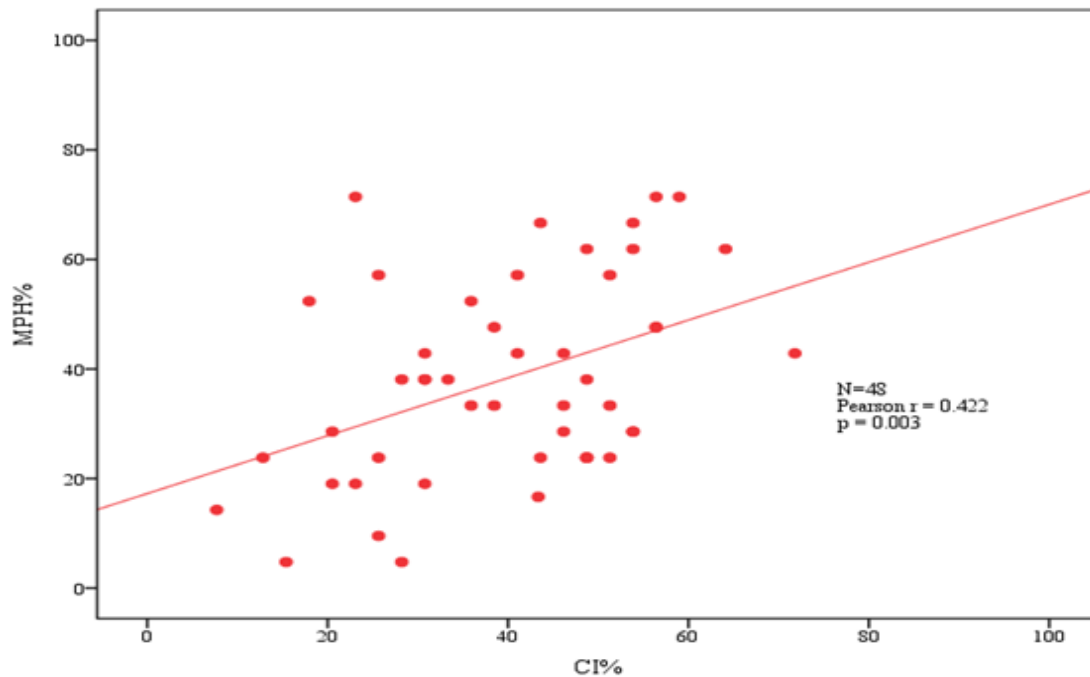


Figure 2-5 Correlation between muscular hyperplasia (MPH) and chronic inflammation (CI) (by global %PMTS)

(Figure adapted with permission from Shivaji et al)

2.5 Chronic inflammation and the effect of its location within a fibrotic stricture

In view of the positive correlation with fibrosis that was revealed on detailed analysis, chronic inflammation was looked into more closely, in our cohort. One interesting finding noted was that patients who had chronic inflammation predominantly within the submucosal and muscularis propria layers (as per %PMTS taken as average) i.e., deeply penetrated changes, appeared to have a more aggressive course of disease, mostly in the form of recurrence, requiring further surgery or significant disease activity requiring escalation to biological therapy. The number of patients was not large enough to draw conclusions based on statistical significance within this cohort.

When CI scored higher in the mucosa, the disease course was not as aggressive, and they were

more likely to achieve remission. Twelve patients who had the highest scores for CI predominantly in the submucosa and muscularis propria (as per %PMTS taken as average) had their recent follow up data reviewed. These patients were divided into tertiles in descending order of the %PMTS. Details of individual scores and their outcome on most recent follow up visit are given below.

(Table 2-6)

Table 2-6 Tertiles (based on descending order of average scores of chronic inflammation within the submucosa and muscularis propria) and outcomes in these patients. The patients in the highest tertile for chronic inflammation had adverse disease course post-surgery

	%PMTS for Chronic inflammation		Average %PMTS	Outcome on follow up Median follow up 45 months; range 24-60 months
	In SM	In MP	Average score	
Tertile 1 (Highest average scores for CI)	78	67	72.5	Recurrence and surgery On biologics now
	78	67	72.5	Recurrence and surgery Active disease now
	56	78	67	Aggressive fistulising disease On biologics now
	67	56	61.5	Recurrence and surgery On 2nd biologic
Tertile 2 (Moderate average scores for CI)	78	44	61	Recurrence with active disease On thiopurines now
	78	44	61	Recurrence and surgery Now in remission
	78	22	50	In remission – not on any therapy
	67	44	55.5	Recurrence and active disease In remission with biologics
Tertile 3	67	44	55.5	In remission- not on any therapy
	67	33	50	In remission- not on any therapy

(Low average scores for CI)	56	33	44.5	In remission on thiopurines
	56	22	39	In remission on thiopurines

2.6 Discussion

We found in our study that resected Crohn's related strictures are not purely fibrotic even if surgery was influenced by pre-operative categorisation of stenotic segments as fibrotic & non-inflammatory. Our results show that strictures are composed of several components that contribute towards narrowing of the bowel lumen, in particular, chronic inflammation that exists in all specimens. Once this was noted, there was further focus on chronic inflammation as a component of interest. We found that it also appears to have an effect on clinical outcomes varying based on which layer of the bowel wall this is found within.

In an exploratory analysis, it was noted that patients whose strictures scored high for chronic inflammation in the submucosa and muscularis propria appeared to do worse than others during post-surgical follow-up period. In light of our findings, whether fibroblasts (and myofibroblasts), when activated were able to continue to drive chronic inflammation within the microenvironment of the bowel wall became a pertinent question.

These findings are somewhat different compared to what was found in the previous cohort where this same scoring scheme was applied. The patients at University of Calgary were found to have smooth muscle hyperplasia/hypertrophy to be the most prominent component within a Crohn's related stricture, although chronic inflammation was also prominent. One possible explanation is the proportion of patients on biological therapy in both groups. In the Canadian cohort, all

patients were on biologics with anti-tumour necrosis factor alpha (TNF α), just prior to and leading up to resection. In the Birmingham cohort a small number of patients had been exposed to biologics. Whether this is the main reason that led to increased inflammation component is speculative but noteworthy.

The histological phenotypes seen in the Canadian cohort have been reported previously, in patients receiving anti-TNF therapy for CD.(Schaeffer, Walsh et al. 2014) The study from Calgary also showed that in addition to collagen deposition related-volume expansion within the bowel wall, adipose hyperplasia and volume expansion of extra cellular matrix (ECM) due to enrichment of mesenchymal cells also played a role.(Chen, Lu et al. 2017) Intestinal stricture formation in CD is a complex process with multiple pathways likely to be involved in fibrosis, (Rieder and Focchi 2009) and also appear to be distinct compared to conventional fibrosis. This process is triggered by intestinal damage activating several lines of cells in order to help maintain gut integrity and promote healing. These cells usually derive from resident mesenchymal cells (fibroblasts, sub-epithelial myofibroblasts, and smooth muscle cells).(Latella, Di Gregorio et al. 2015) In the gut, these are also recruited from epithelial and endothelial cells (via epithelial-mesenchymal transition [EMT]/endothelial-mesenchymal transition [EndoMT]), stellate cells, pericytes, and bone marrow stem cells.(Latella, Di Gregorio et al. 2015)

Our findings reveal some new interesting features. We noted that some components in the bowel wall have a stronger correlation with fibrosis compared to others; components such as neuronal hypertrophy and adipose hyperplasia may also have an important role to play but the significance of these remains uncertain at present. The impact of neuronal hypertrophy on disease course and outcome was also looked into, but in our cohort of patients, there appeared to be no significance of this component.

The statistically significant correlation between chronic inflammation and fibrosis is an important finding. Our results show that 'pure' fibrosis is unusual and chronic inflammation is a constant presence in all strictures. This would suggest, as mentioned in other studies(Wynn and Ramalingam 2012), that some of the pathways and mechanisms responsible for a sustained inflammatory state may also promote or trigger changes that lead to fibrosis. Some of the important pro-fibrotic mediators that have been identified include TGF β , activins, integrins, connective tissue growth factor (CTGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF)-1 &-2, epidermal growth factor, fibroblast growth factors (FGFs), endothelin 1, 2 & 3, cytokines like interleukins (IL) 1,4,6,13, 17, 21, 22, 23 33. 36. Some of the mediators with anti-fibrotic properties include interferons (IFN- α , IFN- γ), IL-7, IL-10, IL-12, Smad7 and nitric oxide.(Wynn and Ramalingam 2012, Latella, Di Gregorio et al. 2015) These mediators are known to remain at high levels during a sustained inflamed state. Once activated, it is very likely that fibroblasts, myofibroblasts and smooth muscle cells contribute to fibrosis, as well as volume expansion leading to stricture formation,(Rieder, Zimmermann et al. 2013) and possibly inflammation. When the balance between pro & anti-fibrotic mediators is probably lost, this causes intestinal fibrosis.

Inflammation and fibrosis are closely related. There is likely to be an interplay between the two and it has been recognized that inflammation is probably one of the main drivers of fibrosis not only in CD but also in ulcerative colitis (UC). A recent study published in 2018 looking at patients undergoing colectomy for refractory ulcerative colitis (UC) found that there was significant degree of fibrosis in the muscularis layer of colectomy samples.(Gordon IO 2018) They noted submucosal fibrosis in 100% of UC colectomy specimens in areas affected by inflammation. Submucosal fibrosis was associated with the severity of intestinal inflammation ($r=0.58$; $P < 0.001$) and

histological changes of chronic mucosal injury.(Gordon IO 2018) It has also been noted that in some patients with CD there was improvement in strictures or delay in formation of strictures by effective control of inflammation with biologics and immunomodulator therapy (Safroneeva, Vavricka et al. 2015) but there is conflicting evidence for this observation.

It has been proposed that some patients with a non-inflamed bowel also go on to develop fibrosis. The extracellular matrix (ECM) laid down by activated mesenchymal cells acts as a storage site for cytokines and fibrotic growth factors, including TGF- β . The release of these promotes deposition and crosslinking of ECM components which could change its local mechanical properties, making it stiffer. The stiffness by itself could further stimulate fibrogenesis by activating mesenchymal cells, even in the absence of other activating factors.(Rieder, Fiocchi et al. 2017) The mesenchymal cells could further increase stiffness independent of inflammation, thus perpetuating the cycle.

Our study gives an insight into the predominant components of a stricture. Like some studies have reported previously, our results challenge the conventional knowledge that most strictures requiring surgery are due to collagen deposition and fibrosis. From the histological point of view, no Crohn's stricture is simply "fibrotic", and the traditional fibrotic versus inflammatory classification is not supported by histological analysis. To turn our focus back to control of chronic inflammation would be a more reasonable strategy in medical treatment.

We have also been able to show statistically significant correlations between various components. The knowledge that muscular hyperplasia, volume expansion and adipose hyperplasia all occupy significant space in a stricture should trigger further work into looking at these as potential targets of therapy.

2.7 Limitations of the study

This study has certain limitations. This was a cohort selected in a single tertiary centre where the patients attending with IBD are more likely to have a severe disease course. This was a retrospective study design and the data collected was not as robust due to some missing fields. The number of patients who underwent surgery was perhaps lower than expected in a tertiary care centre, as this study was undertaken prior to the LiRIC trial. (Ponsioen, de Groof et al. 2017) The role of surgery has evolved since 2018 where there was a paradigm shift in managing patients with limited terminal ileal disease of (<40cm).

We also noted that the uptake of biological therapy in our cohort at the time of or just prior to resection was low. It is unclear if biologics would have had an impact on the changes seen histologically in these patients. There are studies which have shown that prior or current biological therapy is a risk factor for surgery in CD.(Bossuyt, Debeuckelaere et al. 2018) This could probably be explained by an aggressive disease phenotype or by sub-optimal control of inflammation owing to histological location, such as seen in our cohort. This is yet unclear and is a subject requiring further investigation. However, it is possible that the disease trajectory could be slightly modified in patients exposed to biologics. This has been shown by Schaeffer et al where they compared histology of resections from patients with CD exposed to anti-tumour necrosis factor (anti-TNF) therapy undergoing surgery, to those not exposed to anti-TNF.(Schaeffer, Walsh et al. 2014) They reported that acute & chronic inflammation in the mucosa and submucosa were lower in the anti-TNF exposed group; these patients had significantly higher reduplication of muscularis layer along with a distinctive fibrosis at this level.(Schaeffer, Walsh et al. 2014) Although this study was done using descriptive analysis of histology, the changes in the anti-TNF

exposed group reported are generally similar to patients in our Calgary cohort. We have not analysed the bowel histology proximal to the stricture in our study, but this has been done in our previous study.

The finding of chronic inflammation in the deeper layers and deep ulcerations (**see Figures 2-6, 2-7 & 2-8**) may be driving pathways that promote fibrosis within the microenvironment which is unlikely to be affected by biological therapy. This is an important point to note, as any anti-fibrotic therapy used in the future may need to be used in adjunct with effective anti-inflammatory therapy for sustained benefit to break the vicious cycle of chronic inflammation and fibrosis, particularly mediated by TGF- β .(Ihara, Hirata et al. 2017)

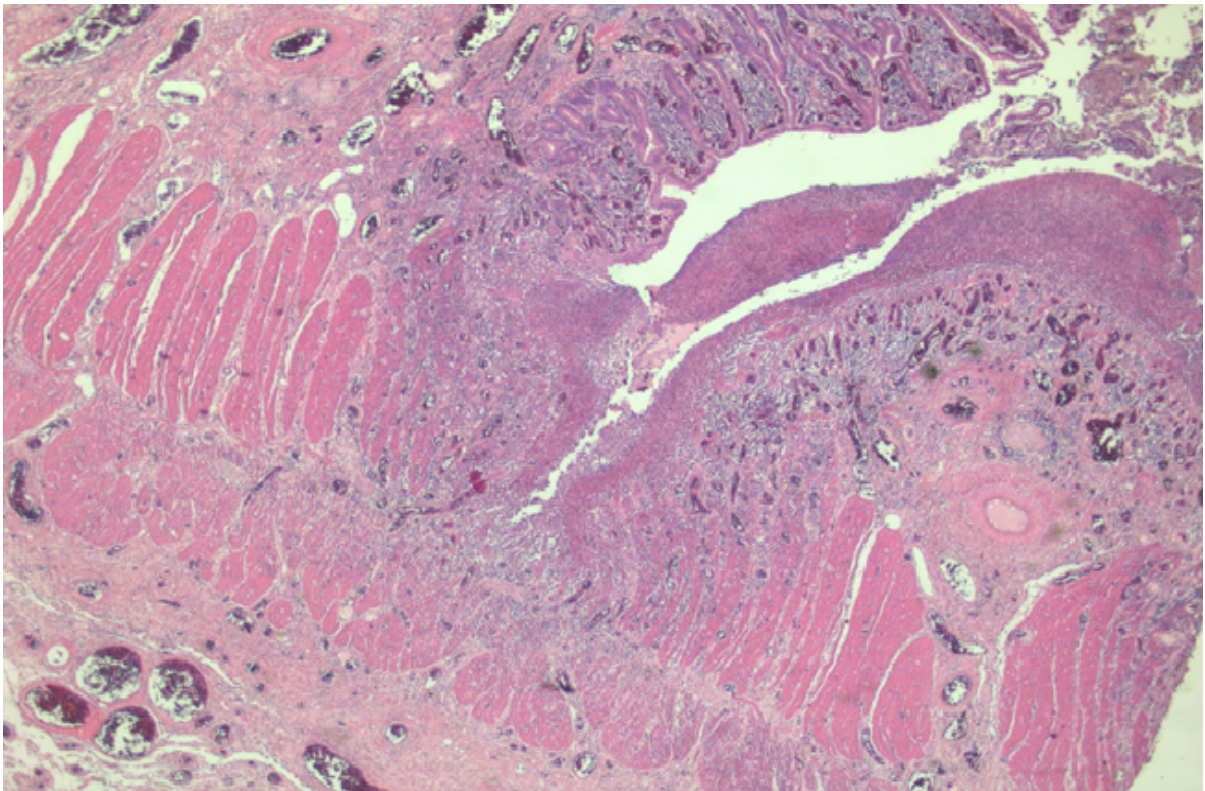


Figure 2-6 Haematoxylin and eosin section (x1.6) displaying fissuring ulceration

This section shows fissuring ulceration involving the submucosa and muscularis propria, which shows muscular hypertrophy. There is no adipocyte proliferation in the submucosa in this case. The fissure is extensive, involving mucosa, submucosa and muscularis propria. This was assigned a score of 3, severe, for fissuring ulcer as part of inflammation scoring, on each of these layers
(Figure adapted with permission from Shivaji et al)

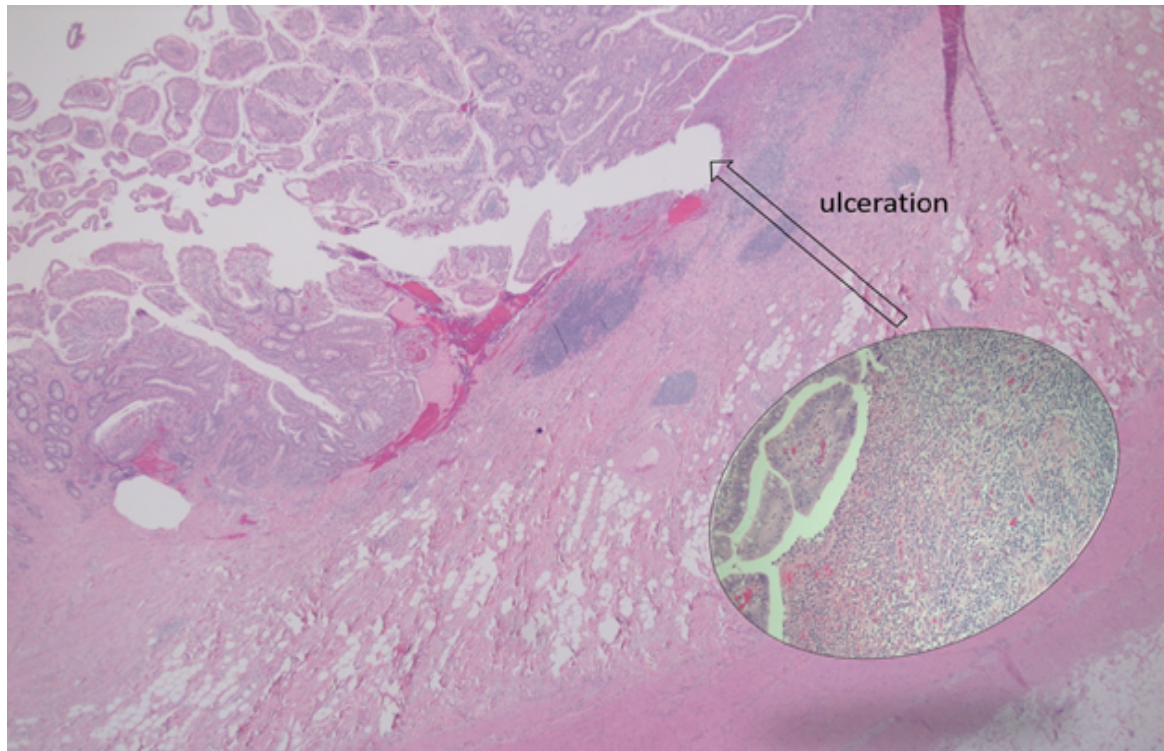


Figure 2-7 Haematoxylin and eosin section (x1.6) displaying a superficial ulcer (inset x10). Section of the strictured segment of small intestine showing mucosal ulceration (high power of the ulcerated field in inset)

This case shows widespread adipocyte proliferation in the submucosa associated with space moderate volume expansion. The ulcer was assigned a score of 2, moderate for superficial ulceration as part of inflammation scoring, on mucosa. Adipocyte proliferation is noted in the submucosa, which was assigned a score of 2, moderate as part of the adipocyte proliferation score on submucosa (dark arrows).

(Figure adapted with permission from Shivaji et al)

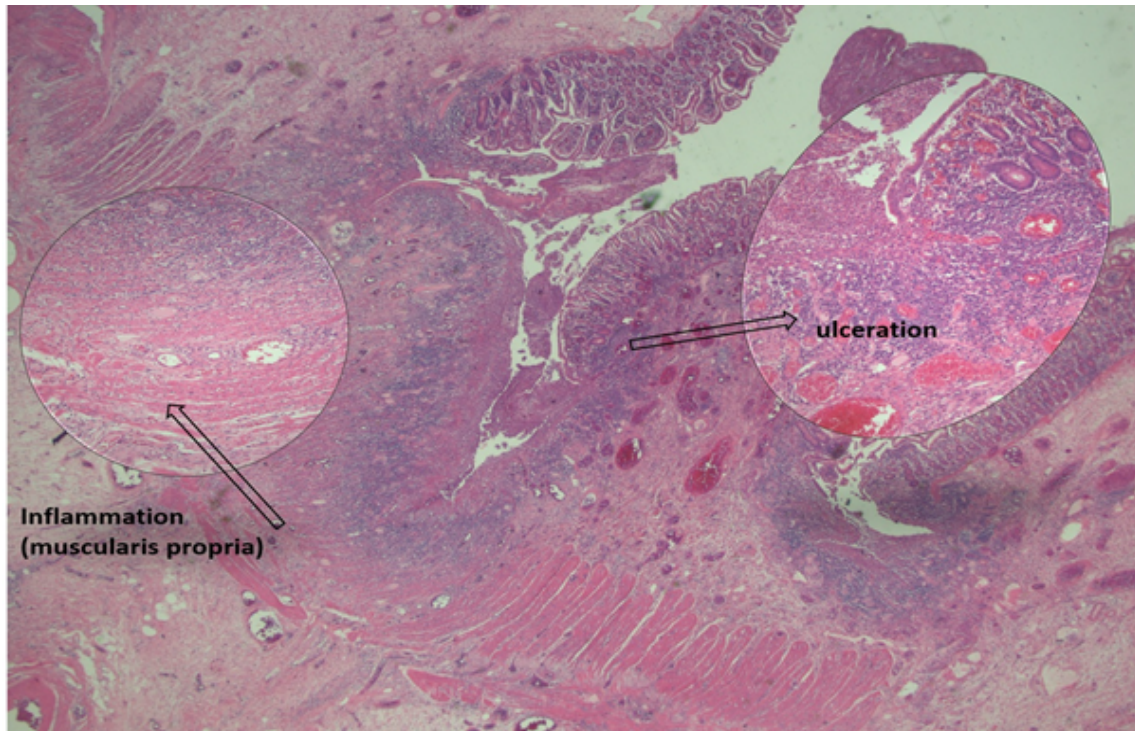


Figure 2-8 H&E section of the strictured segment showing mucosal ulceration (inset) extending into the submucosa and muscularis propria (inset)

Muscular hypertrophy is more evident in the longitudinal layer. The subserosal adventitia is inflamed and fibrotic. The fissuring ulcer (inset x10) is seen to involve muscularis propria (inset x10). There is no significant adipocyte proliferation on this section, which would have attracted a score of 0, none, as part of the adipocyte proliferation score on submucosa (dark arrows). *(Figure adapted with permission from Shivaji et al)*

2.8 Radiology as a diagnostic and predictive tool in patients with chronic inflammation

Radiology is an important non-invasive diagnostic tool in investigating and monitoring IBD. The types of scans available to use in IBD along with their pros and cons have been discussed in section 1.1.4.3. In this study, we were interested in looking into radiology as a predictive tool in patients with stricturing CD. To fulfil this objective, in our cohort, we undertook a sub-group analysis for those patients who had an MRE scan within 6 months preceding surgery. There are multiple scoring systems used and validated in IBD. (Rozendorn, Amitai et al. 2018) After internal discussions, we selected a validated scoring system to methodically score the MRE scans in retrospect. We selected the Crohn's Disease MRI Index (CDMI) score as it has been validated against histology and is reasonably user-friendly. (Steward, Punwani et al. 2012) The CDMI is also considered to be less cumbersome to use compared to other systems. The scoring system grid for the CDMI score is given below in **Figure 2-9**.

Definitions of the qualitative MRI activity index.				
Score	0	1	2	3
Mural thickness ^a	1–3 mm	>3–5 mm	>5–7 mm	>7 mm
Mural T2 signal ^b	Equivalent to normal bowel wall	Minor increase in signal-bowel wall appears dark grey on fat saturated images	Moderate increase in signal-bowel wall appears light grey on fat saturated images	Marked increase in signal-bowel wall contains areas of white high signal approaching that of luminal content
Perimural T2 signal	Equivalent to normal mesentery	Increase in mesenteric signal but no fluid	Small fluid rim (<2 mm)	Larger fluid rim (>2 mm)
Mural enhancement pattern	N/A	Homogeneous	Mucosal	Layered
Enhancement ^b	Equivalent to normal bowel wall	Minor enhancement – bowel wall signal greater than normal small bowel but significantly less than nearby vascular structures	Moderate enhancement – bowel wall signal increased but somewhat less than nearby vascular structures	Marked enhancement – bowel wall signal approaches that of nearby vascular structures
Lymph nodes	Absent	Cluster less than 1 cm	1 node greater than 1 cm	3 nodes greater than 1 cm
Lymph node enhancement ^c	Less than nearby vascular structure	Equivalent or greater to nearby vascular structure		
Comb sign	Absent	Present		

^a Measured using electronic calipers.
^b Compared to normal small bowel.
^c Compared to nearest vessel.

Figure 2-9 Crohn's Disease MRI Index (CDMI) scoring system

In this scoring system, the first step is to record the initial features, for which a numerical score is assigned. To arrive at the total CDMI score, a formula is applied - $1.79 + 1.34 \times \text{mural thickness} + 0.94 \times \text{mural T2 score}$.

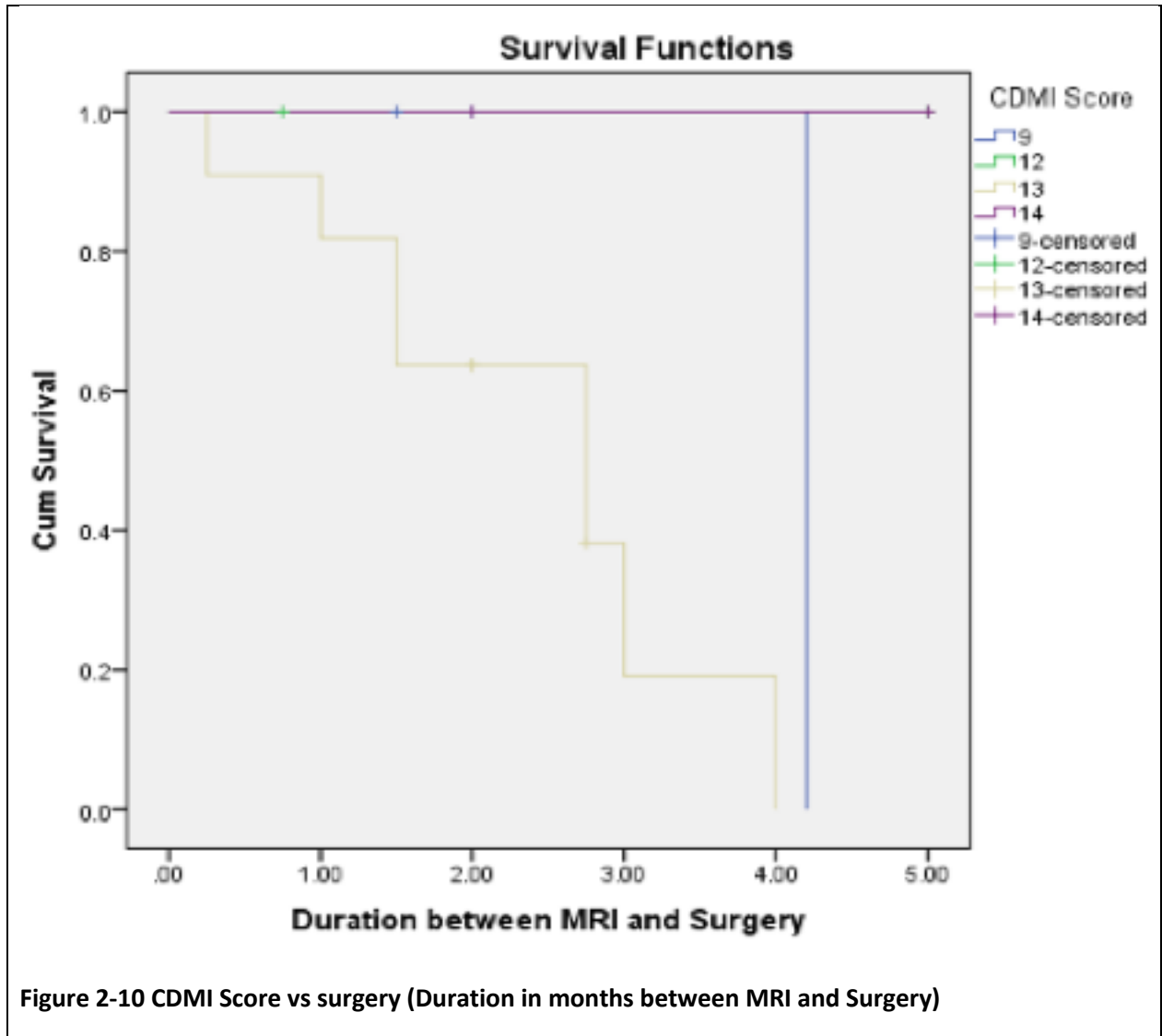
If the final score is ≥ 4.1 , this is indicative of active disease.

Of the 48 patients, 25 had an MRE within the preceding 6 months of surgery. The scans were scored using the CDMI system by a senior radiologist with a subspecialty interest in IBD. The radiologist was blinded to patient details and outcomes.

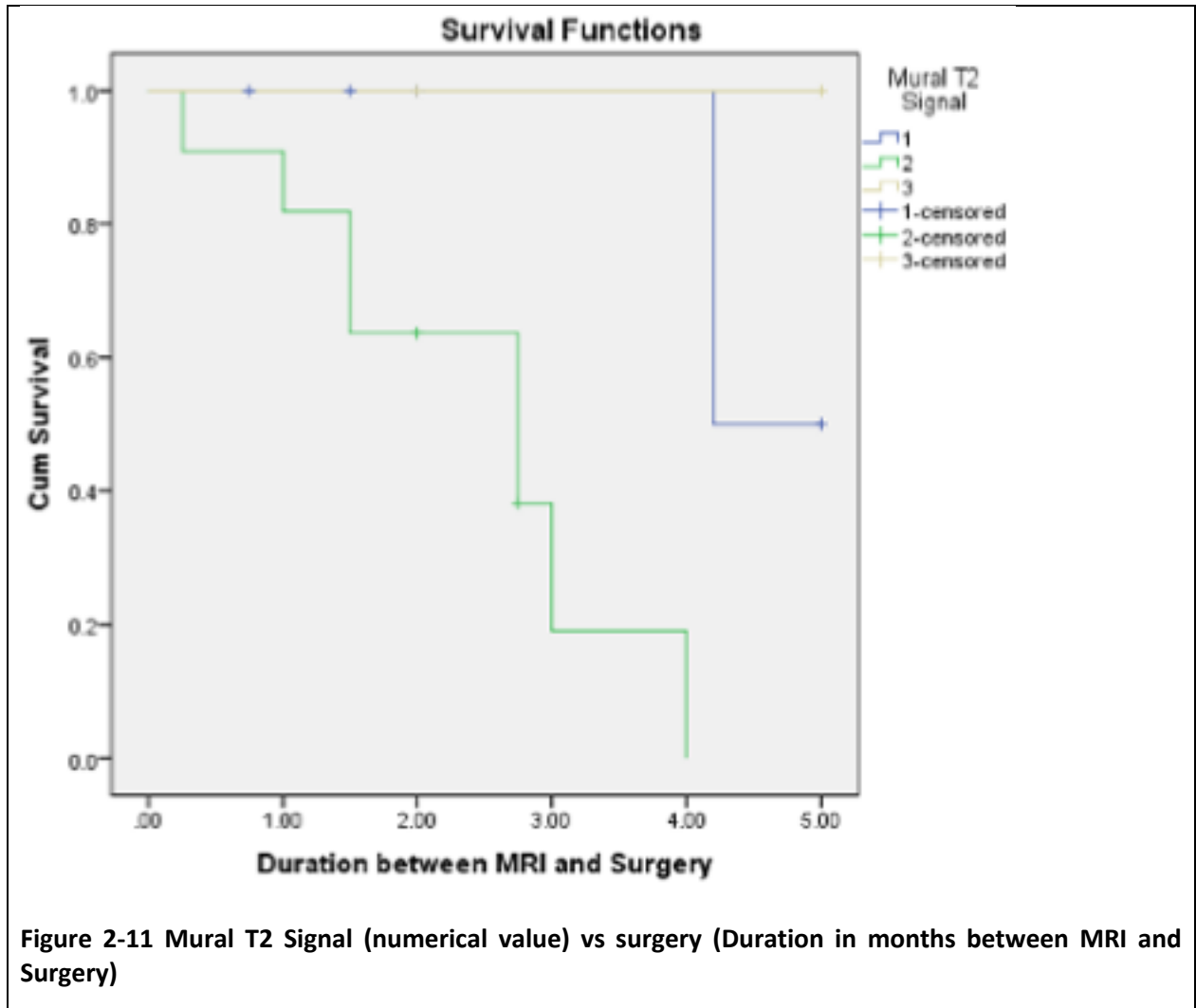
Statistical analysis of radiology and clinical outcome

All statistical analysis and graphs were generated using the IBM® SPSS®, (version 20) statistics software. For the Kaplan-Meier (K-M) Survival analysis, each patient was categorised using three variables (i) Time interval between MRI and surgery (event to predict) (ii) their status at the end of the study follow-up period (event occurrence or censored) and (iii) the study group they are in. The test of equality of survival distributions for the different levels of the MRI parameters (e.g., total CDMI score, Mural T2 signal, Mural thickness, Mural enhancement pattern, perimural T2 signal, lymph node enhancement, and comb sign) vs. patient event or outcome, was analysed using Log Rank (Mantel-Cox) test.

On analysis, we noted that all our patients had active disease on MRE as per the CDMI scoring. Also, patients with a high CDMI score were more likely to undergo surgery within 4 months of the MRE. A score of greater than 12 was predictive of requiring surgery and this was statistically significant ($p=0.042$). This is represented in a K-M survival curve (**Figure 2-10**).



When we performed another analysis on the individual weighted scores, only the mural T2 signal (numerical value of 2 or more) was predictive of surgery in the next 4 months. This was statistically significant ($p=0.02$). This is represented in a K-M survival curve. **(Figure 2-11)**



2.9 Conclusions

In conclusion, we demonstrated in our study using a grading system that histology of a stricture in CD comprises of several components such as chronic inflammation, muscular hyperplasia and volume expansion in addition to fibrosis itself. There is a strong and statistically significant positive correlation between chronic inflammation and fibrosis. Even when a stricture is identified as fibrotic and resected, chronic inflammation remains a prominent component of the stricture.

Purely anti-fibrotic approach in future may not be successful without targeting the inflammation as well. Chronic inflammation when predominantly seen in the submucosal or muscularis propria possibly points to a higher risk of recurrence and further surgery.

In addition, we also demonstrated that MRI could be a very strong and accurate tool in predicting surgery in this cohort of patients. The CDMI score is reliable in identifying active disease, and correlates well with histology. It could be used for risk stratification to identify patients at high risk of surgery. This could prove very helpful in an MDT setting to aid clinical decision making.

2.10Future directions

The findings from this retrospective study provided the foundation on which a prospective study was designed to explore the immunological factors driving chronic inflammation, and its effects on intestinal fibrosis.

The findings on radiology are also worth exploring. The use of CDMI could be considered as a reliable option to predict surgery in this group of patients. It could perhaps be embedded in the reporting software for ease of use.

3 CHAPTER 3: STUDY OF CLINICAL OUTCOMES IN PATIENTS DUE TO SUBOPTIMAL CONTROL OF INFLAMMATION

Parts of this chapter (section 3.1) have been orally presented at the British Society of Gastroenterology Conference 2022 and available online-

Abhishek Rao, Alina Bazarova, Pathrabali Mitra, Snehal Majumder, Tommaso Lorenzo Parigi, Subrata Ghosh, Marietta Iacucci, **Uday N Shivaji**

-Delayed IBD diagnosis leads to worse outcomes over years, with multiple implications on service delivery. Gut 2022;71:A5-A6. DOI- 10.1136/gutjnl-2022-BSG.9

-Delayed IBD diagnosis increases risk of requiring biologics in first 4-years -an inception cohort study. Gut 71(Suppl 1):A56.2-A57. DOI:10.1136/gutjnl-2022-BSG.99

Parts of this chapter (section 3.2) have been published as-

Shivaji UN, Bazarova A, Critchlow T, Smith SCL, Nardone OM, Love M, Davis J, Ghosh S, Iacucci M. *Clinical outcomes, predictors of prognosis and health economics consequences in IBD patients after discontinuation of the first biological therapy.*

Therap Adv Gastroenterol. 2020 Dec 27;13:1756284820981216 (Shivaji, Bazarova et al. 2020) doi: 10.1177/1756284820981216. PMID: 34104206; PMCID: PMC8162203

Overview of chapter

This chapter includes two major studies undertaken to investigate the impact of poorly controlled inflammation in IBD, and its outcomes in clinical practice. In keeping with our plan to study effects of persistent chronic inflammation, two distinct patient cohorts at different stages of their disease trajectory were selected, to record factors and adverse outcomes. This is discussed in two sections.

Section 3.1

This cohort comprised of inception cohort patients in whom the initial diagnosis of IBD was delayed and therefore inflammation was persistent. This was a treatment naïve group and patients suffered a sustained inflammatory state due to patient-related or health-system related delays. This would give important insights into outcomes in the initial and immediate few years of IBD diagnosis.

Section 3.2

This cohort comprised of IBD patients who were further in their disease journey. They had been on advanced therapy with biologics, but for various reasons, these drugs were withdrawn in these patients. This withdrawal meant a real risk of suboptimal control of inflammation.

In both groups, the suboptimal control of chronic inflammation was the apparent common factor, but at different stages of disease. These real-world studies of clinical outcomes in two different cohorts gave us new useful information into effects of chronic inflammation., that eventually guided us to design the prospective study.

3.1 Delays in diagnosis of IBD

3.1.1 Background

Inflammatory bowel diseases (IBD) can cause significant physical and psychological distress, leading to a diminished quality of life for those affected. Unfortunately, delayed diagnosis of IBD is not uncommon, and it can have profound consequences on patients' well-being and long-term prognosis. (Ghosh and Mitchell 2007)

These delays could be multifactorial. Firstly, the diagnostic journey for patients with IBD often begins with a wide range of nonspecific symptoms, such as abdominal pain, diarrhoea, rectal bleeding, vomiting, weight loss, and fatigue. (Burgmann, Clara et al. 2006) During the initial stages, these symptoms could be erroneously attributed to other more common gastrointestinal (GI) conditions (such as irritable bowel syndrome or GI infections). (Burgmann, Clara et al. 2006) In some cases, they could even be dismissed as a result of stress or dietary factors, until the time they present repeatedly with persistent or worsening symptoms. As a result, patients may experience a protracted period of uncertainty and frustration before receiving a definitive diagnosis. Secondly, healthcare professionals, particularly in the community, face challenges in recognizing and distinguishing IBD from other gastrointestinal conditions. The location and type of disease could also have an impact on delayed diagnosis. (Vavricka, Spigaglia et al. 2012) The IOIBD group tried to address the issue of delays by studying the red flags to help diagnose CD earlier. (Danese, Fiorino et al. 2015) Based on a comprehensive survey of doctors, CD patients and non-CD patients, they reported eight parameters that were statistically accurate in helping with a quick diagnosis of CD. This could serve as an important tool in distinguishing IBS from IBD

but needs to be validated before it is used routinely in clinical practice. (Danese, Fiorino et al. 2015)

The lack of specific and easily accessible diagnostic tests such as endoscopic procedures and imaging studies also result in a delay. Thirdly, in addition to clinical hurdles, delays in diagnosis can also result from patient-related factors. Some patients may delay seeking medical attention due to fear, embarrassment, or a belief that their symptoms are transient and will resolve on their own. Others may face genuine barriers in accessing healthcare services, such as long wait times for appointments or limited access to gastroenterologists. The conventional patient journey within the community and primary care and the process within a hospital setting after referral by GP are illustrated in **Figures 3-1 and 3-2**.



Figure 3-1 Conventional patient journey in the community and primary care

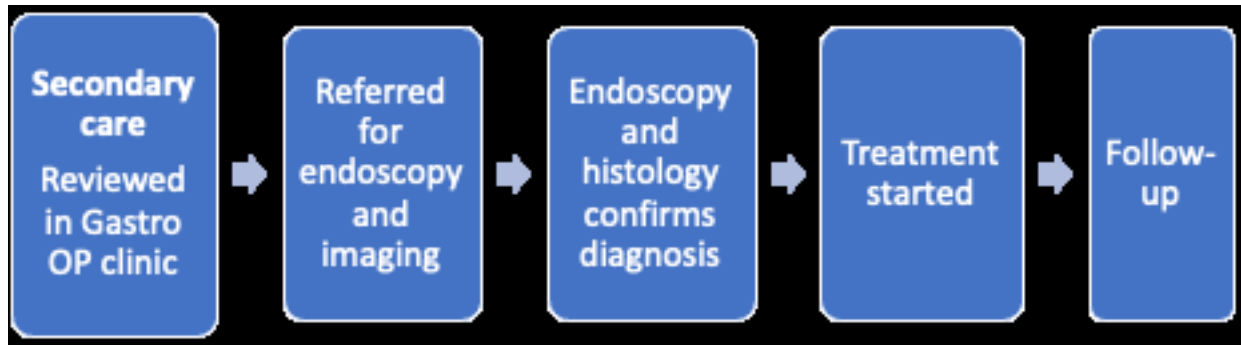


Figure 3-2 Conventional patient journey in the hospital

The consequences of delayed diagnosis of IBD can be far-reaching. Chronic untreated inflammation can lead to complications like intestinal strictures, abscesses, and fistulae. (Moon, Jung et al. 2015), necessitating more aggressive treatment approaches. Patients are more likely to require biological therapies and intestinal surgery due to delays. (Lee, Koo et al. 2017, Kang, Koo et al. 2019) Delays may also result in later initiation of appropriate therapeutic interventions, which could further worsen disease progression and risk of long-term complications. It is possible that the increased rates of adverse events are secondary to a sustained state of chronic inflammation. As most of these patients are treatment-naïve, non-resolving CI continues with detrimental effects.

Aside of the physical complications that patients endure, the psychological impact of delayed diagnosis cannot be overlooked. The uncertainty and anxiety experienced by patients during the

prolonged diagnostic journey can significantly impact their mental well-being, leading to increased stress, depression, and reduced overall quality of life. (Sainsbury and Heatley 2005)

With growing evidence that delayed diagnosis has a detrimental effect in both UC and CD, (Jayasooriya, Baillie et al. 2023) it is crucial that the issue is addressed effectively to improve patient care and clinical outcomes. In order to achieve this, it is important to understand the factors causing delays, which may be different from one hospital/health-system to another. Once these have been identified, including the various points or bottlenecks of delay, the appropriate measures can be taken to address them. In general, they require addressing both health-system related and patient-related factors.

3.1.2 Aims of the study

The time leading up from onset of symptoms to formal diagnosis of IBD is considered a delay if the duration is beyond accepted timelines. This time interval is a surrogate marker for sustained CI, leading to a hypothesis that a delay is likely to result in worse clinical outcomes. Unfortunately, delays are common in the health system due to various factors and inevitably the patients are left untreated.

The aims of this study were to understand the impact of delays in diagnosis of IBD and report on IBD-related adverse outcomes (AOs). In this retrospective study, we aimed to study the following-

- To record any delays faced by newly diagnosed IBD patients at our centre
- If so, to understand the various points at which there are delays and identify bottlenecks
- To understand the factors leading to delays i.e., patient-related factors, community-related factors, or hospital-related factors

- To understand if the clinical outcomes were worse in patients who faced a delay compared to those who did not.

3.1.3 Patients and Methods

All new patients referred by the General Practitioner (GP) in Primary Care (community health care) over a 5-year period (January 2013 to December 2017), to the Gastroenterology services at our tertiary care centre, for a suspected diagnosis of IBD, were identified using Electronic Medical Records (EMR). All relevant demographic and clinical information were collected using hospital EMR.

For purposes of the study, different delay-types were defined, and respective cut-off times were assigned by investigators for each delay-type. The delay-types were initially considered for each point of visit. Eg.- delays in radiological scan and reporting. However, during interim analysis we modified our approach to understand delays prior to referral to hospital and then delays within the hospital. This would allow a reliable analysis of the data. The cut-off times were assigned based on best average hospital waiting times for IBD benchmarking in the National Health Services, UK (NHS). (Hawthorne, Glatter et al. 2022) We considered reasonably stringent cut-offs given that our centre is a tertiary care with a large, dedicated service for IBD patients. The definitions of delay-type and the details of cut-off times are in **Table 3-1**. All primary care referral letters and clinic letters in hospital were scrutinised for each episode for all included patients. The reasons for delays in patient journey until start of treatment, and data on pre-defined AOs (steroid & other rescue therapies, hospitalisation, surgery including perianal, and death) were recorded for each patient until end of June 2021.

Table 3-1 Definition of delay-type and cut-off times for each type

Type of delay	Cut-off time
Delay in patients seeking medical attention in community (Reflects late self-presentation to GP)	1 month
Delay in GP referral to the point of review in Gastroenterology clinic (Reflects waiting list for outpatient appointments in hospital)	1 week
Delay between first specialty review and index endoscopy (Reflects waiting list for endoscopy)	4 weeks
Delay between first formal diagnosis to start of treatment for IBD (Reflects local guidelines and practices in hospital)	4 weeks

Statistical analysis

The data were analysed using multiple Pearson correlations and Cox proportional Hazard model to determine whether there was a difference in survival without AOs between patients with and without a delay. We tested the significance of each delay and performed Kaplan-Meier (Kaplan 1958) and Cox proportional hazards (Cox 1972) survival analysis to compare patient outcomes when there was a delay versus no delay (as per pre-defined cut-off values).

Ethical considerations

This study was registered with hospital research governance & ethics committee and was given approval to proceed in July 2019. (*Reference number CARMS-15813*)

3.1.4 Results

Among a large number of referrals from the GP to the specialty, patients who underwent investigations in our centre and reached a final definitive diagnosis of IBD were first screened. We

then applied stringent criteria for patient selection (for e.g.- pre-existing diagnosis or new to area IBD patients were excluded) and only newly diagnosed IBD patients were included.

A total of 105 patients were identified (M=58; median age=32y), with a long median follow-up of 55 months (range 5-72m). The demographics of patients are in **Table 3-2**. The most frequent presenting complaints were abdominal pain (44, 41,9%), loose stools (40, 38,1%), bloody diarrhoea (37, 35,2%) and bleeding per-rectum (33, 31,4%) and only 16% declared a family history. 65, 27 and 13 patients had final diagnosis of Ulcerative colitis, Crohn's disease and IBD-unclassified respectively, and analysed collectively. The breakdown of clinical details re in **Table 3-3**. The number of patients with durations of follow-up is illustrated in a plot graph. (**Figure 3-3**)

Table 3-2 Demographics and classification

Age & gender	N=105
Median age	32 years (range 21-82 years)
Gender	Male=58; Female=47
Race	
Caucasian	65 (62%)
Asian	28 (27%)
Afro-Caribbean	1 (1%)
Unknown/Unreported	11 (10%)
Disease type at first diagnosis	
Ulcerative colitis	65 (62%)
Crohn's disease	27 (26%)
IBD-unclassified	13 (12%)

Montreal classification for CD	
Age	
A1	2
A2	22
A3	3
Location	
L1	7
L2	6
L3	13
L4	0
L3+L4	1
Behaviour	
B1	21
B2	3
B3	0
B1+ perianal	3
Ulcerative colitis extent	
E1	12
E2	36
E3	17

Table 3-3 Clinical data - symptoms, medications at start, and follow-up period

Symptoms at presentation	Number of patients
Abdominal pain	44
Loose stools	40
Loose stools with blood in stools	37
Bleeding per rectum	33
Weight loss	16

Vomiting	5
Medications started at first diagnosis	
Oral 5-ASA	48
Oral Steroids	33
Rectal 5-ASA	32
Rectal steroids	8
5-ASA and steroids	6
Thiopurines	5
Biologics	5
Details of follow-up	
Median follow-up period of cohort	55 months (range 5-72 months)
Patients with first follow-up <6 months	101
Patients with first follow-up 6-12 months	2
Patients with first follow-up 12-24 months	2
Patients with first follow-up >24 months	0

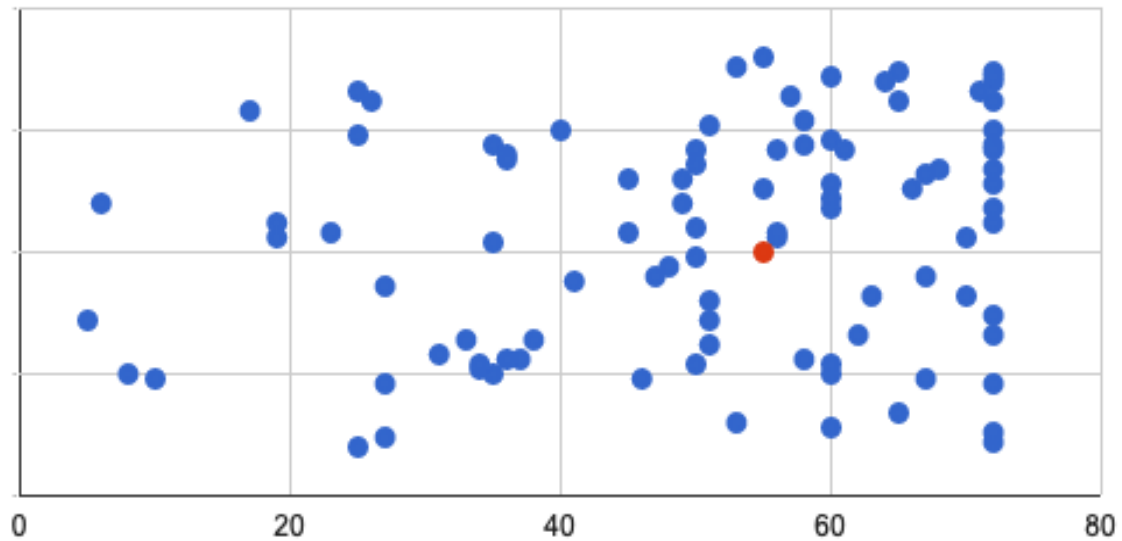


Figure 3-3 Follow up data for all patients (in months)

In this plot, X-axis indicates time in months. The blue dots indicate patient's maximum follow-up at specific time periods. The red dot indicates the median follow-up for the cohort.

In our total cohort, we identified that there were delays at almost every step. The longest delay-types in order were -

- Delay in patients seeking medical attention (median= 4 months; range 1 to 84 months)
- Delay between GP referral to Gastroenterology clinic appointment (median=5 weeks; range 1 to 30 weeks)
- Delay between first specialty review and index endoscopy (median=3 weeks; 1 to 36 weeks)
- Delay between first formal diagnosis to start of treatment for IBD (median=2 weeks; range 0 to 12 weeks)

After patient stratification based on these delay-types and applying the specific cut-off times for each, we noted a statistically significant difference in survival without AOs for all, when comparing delay versus no delay.

When patients delayed seeking medical attention beyond 1 month of onset of symptoms, they were more likely to suffer AOs up to at least 60 months, compared to those who did not delay.

This was statistically significant and is illustrated in **Figure 3-4**.

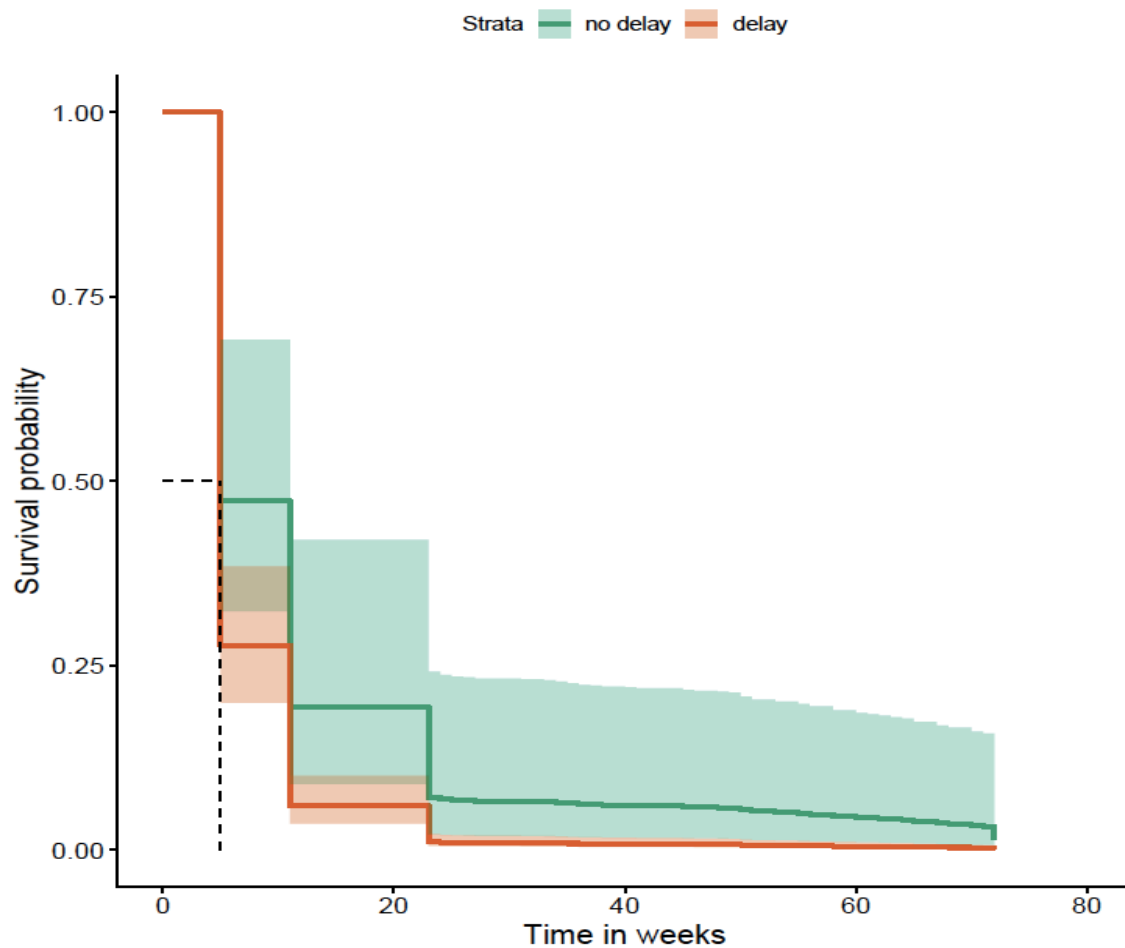


Figure 3-4 Time to seek medical attention of >1month (survival without AOs; $p=0.004$)

The curve illustrates the higher probability of AO due to delay, and the curves remains separated up to follow up period

When patients faced a delay of more than 1 week between referral from primary care to their first specialty review in the Gastroenterology clinic, they were more likely to suffer AOs. This is illustrated in **Figure 3-5**.

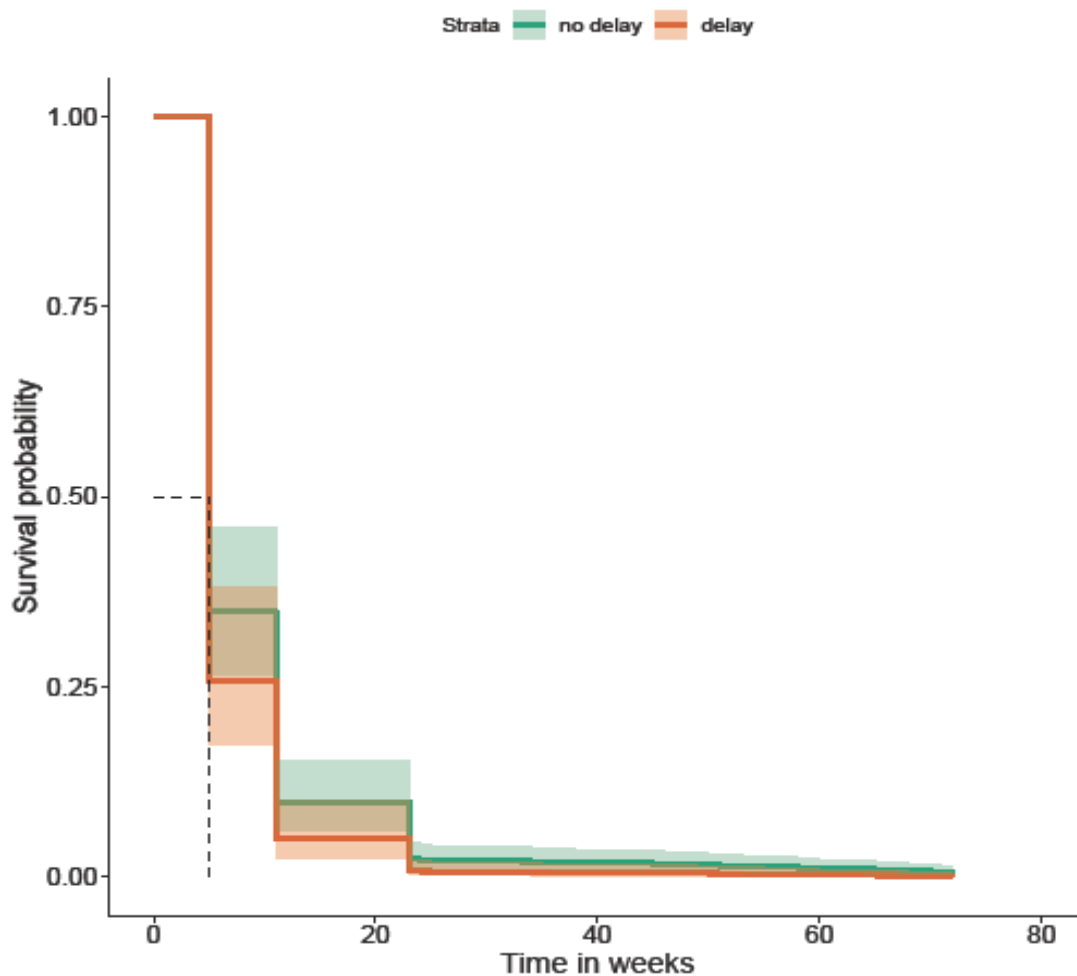


Figure 3-5 Time from GP referral to specialty review of >1 week (survival without AO; $p=0.048$)

The K-M curve illustrates higher probability of AO due to delay

When patients faced a delay of more than 4 weeks within the health system, between their first specialty review and index endoscopy, they were more likely to suffer AOs during the follow-up period for up to 60 months. ($p=0.01$) This is illustrated in **Figure 3-6**.

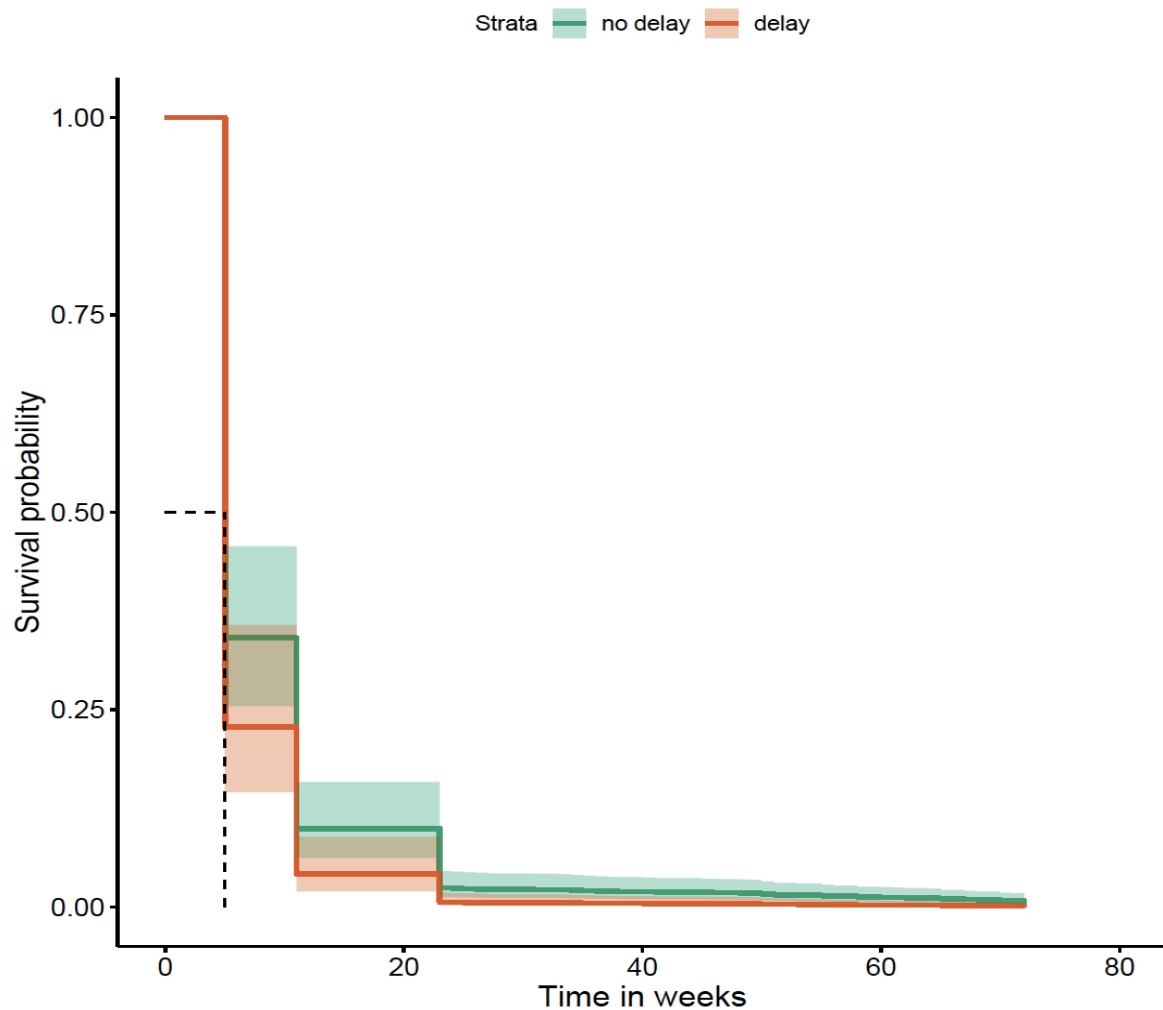


Figure 3-6 Time from clinic review to index endoscopy of >4 weeks (survival without AOs; $p=0.01$)

The K-M curve illustrates the higher probability of AO due to delay

When patients faced a delay of more than 4 weeks between their first formal diagnosis and start of treatment for IBD, they were more likely to suffer AOs during the follow-up period for up to 60 months. ($p=0.03$). This is illustrated in **Figure 3-7**.

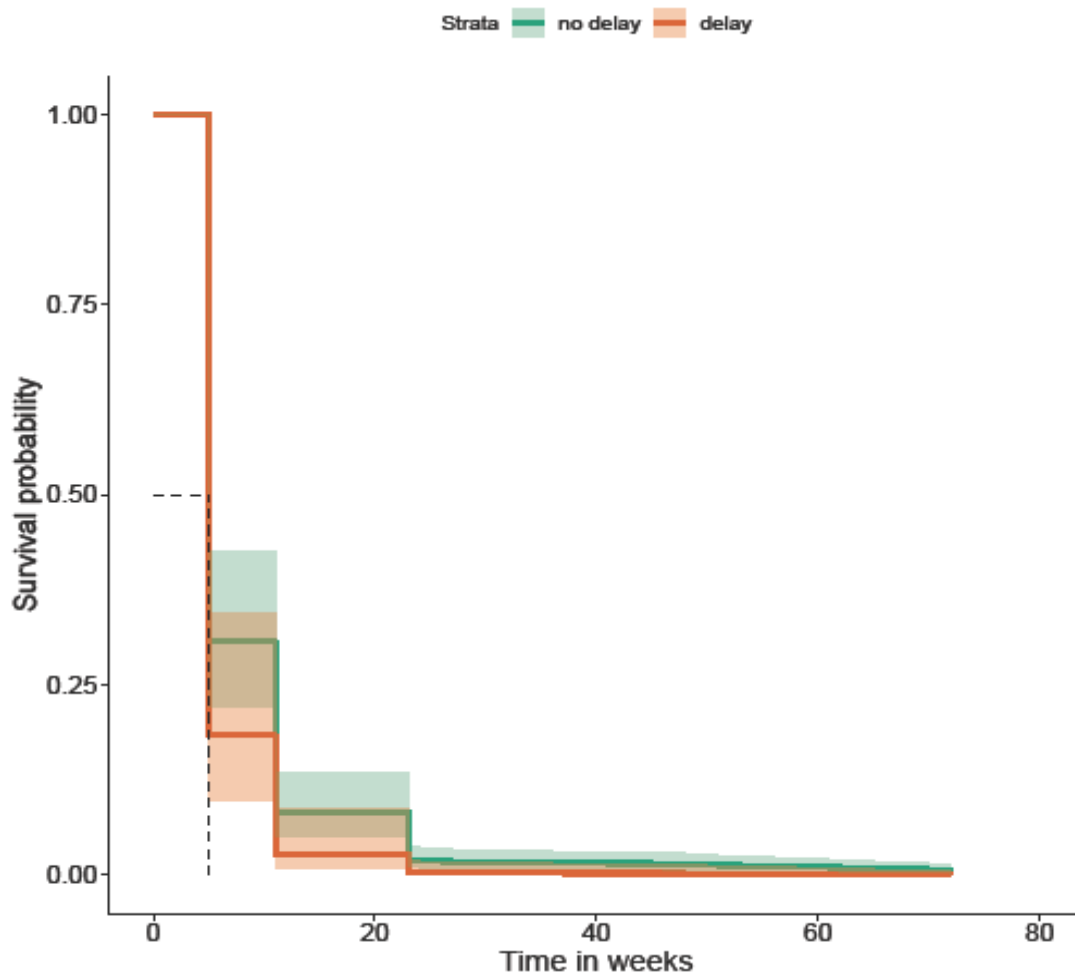


Figure 3-7 Delay between formal diagnosis and start of treatment ($p=0.03$)

The K-M curve illustrates the higher probability of AO due to delay

Delays and biologic use during disease course

During the analysis, we were also interested in examining if delay in diagnosis had an influence on the use of biologics during the follow-up period in our cohort. Using biologic-use at any time during follow-up as an outcome variable, we compared different delay types keeping the cut-off times the same as before. We noted that any delay increased the risk of biologic use during the follow-up period. The details of delay-type and odds-ratio of biologic use is given in **Table 3-4**. Among the patients who started biologics during the follow-up period, majority were started on anti-TNF therapy as their first biologic.

Table 3-4 Risk of biologic use due to delays in diagnosis

Type of delay	Risk of biologic use during follow up (OR with CI)
Delay in patients seeking medical attention in community	1.08 (1.03, 1.17)
Delay between GP referral to the point of review in Gastroenterology clinic	1.10 (1.0, 1.23)
Delay between first specialty review and index endoscopy	1.09 (1.01, 1.21)
Delay between first formal diagnosis to start of treatment for IBD	1.08 (1.0, 1.21)

We also combined more than one type of delay to examine if a cumulative effect was able to predict use of biologics. In our cohort, a model that combined delay in seeking medical attention, and delay between specialty review to index endoscopy exhibited the best predictive power with average AUROC of 0.67.

On further analysis of patient-related delay and health-system related delays, there was a statistically significant increase noted in the risk of requiring biologics at any time during the follow-up period. A patient-related delay of more than 2 weeks showed a statistically significant

increased risk of requiring biologics compared to less than 2 weeks. **Figure 3-8** illustrates this risk, and it is noted that this remains higher for more than 4 consecutive years.

Once patients were within the health system, a cumulative delay of more than 6 weeks increased the risk of biologic therapy over the next 4 years. This is illustrated in **Figure 3-9**.

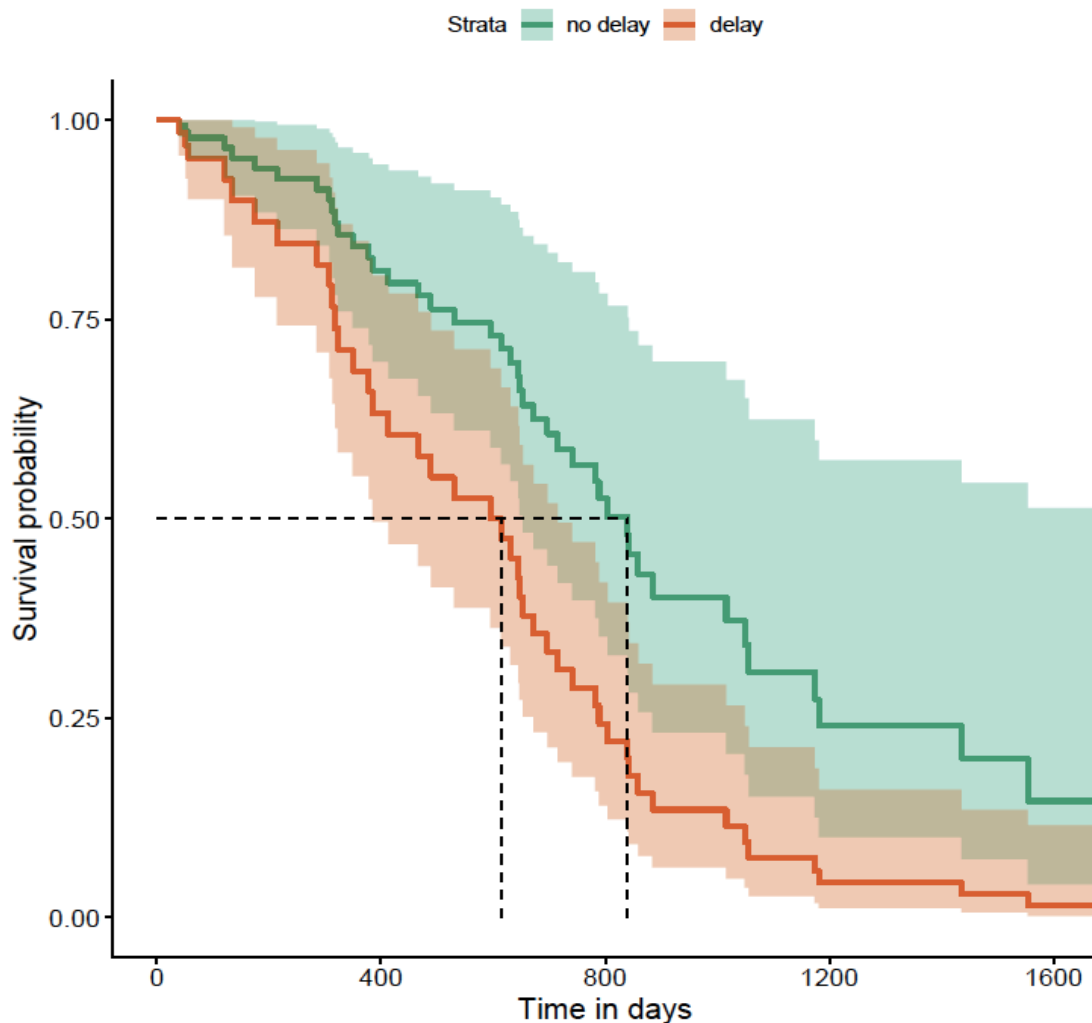


Figure 3-8 Patient-related delay of >2weeks and risk of needing biologics (p=0.042)

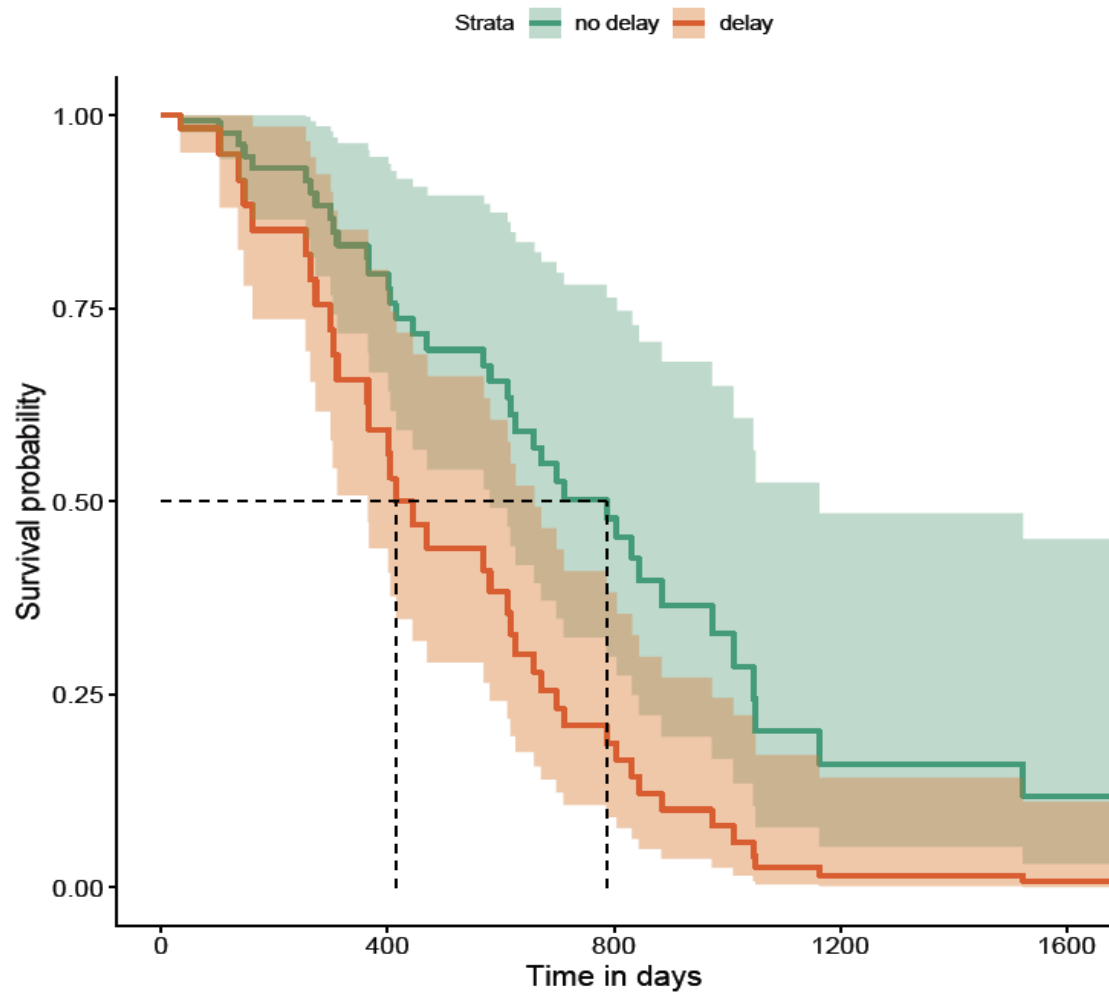


Figure 3-9 Health-system related delay of >6weeks and risk of needing biologics (p=0.021)

3.1.5 Discussion

Delays in diagnosis are unfortunately quite common in most health systems. It is apparent that timely diagnosis is more likely to have a favourable outcome. However, there may be many reasons why timely diagnosis is not always achieved. The poor outcomes due to delayed diagnosis go beyond the obvious perception. There have been multiple studies looking into this aspect, and most have focused on the median delays as per disease types and their outcomes. Our focus in this study was to understand if there is a time scale beyond which the delay becomes detrimental. This topic is particularly important and pertinent now given the significant burdens many health systems are facing during the post-pandemic recovery period. It is important to understand the factors leading to a delay to rectify and offer solutions.

In one study, Vavricka et al reported on risk factors that led to delays in diagnosis of IBD. The authors used data from a comprehensive Swiss national IBD database during a three-year period. They found median delay in CD patients from first onset of IBD-related symptoms to IBD diagnosis was 9 months, significantly longer than UC patients which was 4 months. We found no significant differences between UC and CD. They also reported that younger CD patients with ileal disease were most at risk of long diagnostic delay, probably due to an overlap of IBS-type symptoms. (Vavricka, Spigaglia et al. 2012) In another study, the authors reported similar risk for CD patients. In addition, active smoking status and symptoms onset during summer were found to be independent risk factors. A long diagnostic delay was noted to increase the risk of stricturing

disease (OR, 3.38; $p < 0.01$), fistulae (OR, 2.64; $p = 0.08$), and IBD-related surgery. (Zaharie, Tantau et al. 2016)

In our cohort, for each of the delay-types defined, there were generally more patients who faced a delay compared to those who did not. We were able to compare these within each delay-type.

Delay in patients seeking medical attention

This delay-type which was patient-related was the longest one with a median of 4 months. This is consistent with findings from other studies. (Jayasooriya, Baillie et al. 2023) Although there are prospective studies that have reported a significantly longer median time to diagnosis for CD (Pimentel, Chang et al. 2000), this was not noted in our cohort. For this delay-type, when patients waited beyond a month to seek medical attention, they were more likely to have AOs, and they are more likely to need biologics to manage their condition. In a real-world setting, patients do not always consult the GP immediately for various reasons. Moreover, it remains unclear if a particular timescale (to wait) is acceptable. Based on our findings, it would be reasonable to educate patients not to wait beyond 2-3 weeks to seek medical attention.

Delay between GP referral to Gastroenterology clinic appointment

This delay-type is health-system related, with a median time of 5 weeks in our cohort. In our cohort, a delay of more than 1 week resulted in worse outcomes. This is perhaps the most likely bottleneck that will affect hospitals during the post-pandemic recovery period. There is no reliable data on breakdown of delay-types comparing clinical outcomes. Most studies report on a cumulative delay only. This particular delay reflects the waiting lists in hospitals before a patient is offered an appointment in the specialty clinic. There could be additional pressures due to the

existing cohort of patients needing this service, thereby causing further delays. The waiting time for this critical stage in the patient journey has very likely expanded in almost all healthcare settings due to increasing demand, clearance of backlog of appointments, lack of workforce and shortage of experts. As IBD is a complex disease it is unlikely that treatment is started empirically in primary care before a formal diagnosis can be established. Also, GPs are understandably more comfortable maintaining treatment as advised by experts, than initiating treatment. (Tan, Holloway et al. 2012, Alharbi, Almahmudi et al. 2019) This inevitable delay in starting treatment leaves patients in a state of sustained chronic inflammation, thereby worsening outcomes.

Delay between first specialty review and index endoscopy

This delay-type is the next step in establishing the diagnosis, with a median of 3 weeks in our cohort. It reflects the waiting list in endoscopy departments. Endoscopy is an essential tool not only for diagnosis but also for ongoing assessment of disease activity and monitoring of patients on advanced therapies. Although the median time was less than the assigned cut-off in our cohort, patients who faced delays beyond 4 weeks had a statistically higher risk of having AOs during follow-up. Our data is from pre-pandemic levels and the waiting times have certainly increased since, given the suspension or majority endoscopy activity during the pandemic. (BSG 2020) Studies have shown significant decreases in lower GI endoscopy, including both diagnostic and therapeutic colonoscopy and flexible sigmoidoscopy. (CCUK 2022) As predicted by experts during the pandemic, patients are waiting longer to undergo procedures. (Iacucci, Cannatelli et al. 2020) The increasing waiting lists will leave a larger number of patients at risk of having untreated CI and more AOs.

Delay between first formal diagnosis to start of treatment for IBD

This delay-type generally varies from one hospital to another, based on local policies and guidelines. In our cohort, the median time to start of treatment was 2 weeks which was better than our cut-off of 4 weeks. Among patients who faced a delay beyond 4 weeks, there was a statistically significant risk of AOs. This delay should ideally be minimal as treatment can be started immediately at the time of contact with patients during their index endoscopy. In most cases the clinicians also ask for cross-sectional imaging to reach a complete diagnosis. There are delays at this point too, however we combined this to include it as part of delays within the hospital overall to get reliable analysis. The increasing use of intestinal ultrasound may reduce some of the delays waiting for a CT or MRI scan. (Shivaji, Segal et al. 2022)

As per the pre-pandemic standards of IBD treatment, one study reported more than 75% patients to have started treatment within 2 weeks of diagnosis. (Hawthorne, Glatter et al. 2022) It is possible this proportion may have reduced owing to pressures on the outpatient departments post-pandemic. There have been reports of a drop in prescription rates during the post-pandemic phase. One study reported a sharp fall in prescriptions for thiopurines (-81%), with lesser reductions for biologics (-38%) and oral prednisolone (-20%). (Bodger, Bloom et al. 2021) Any delay at this stage further adds to the already tedious journey the patients go through until the diagnosis is established. This is important to consider as a cumulative delay within the health-system of more than 6 weeks significantly increases the risk of patients requiring biologics over the next 4 years.

It is clear from our data that any delay in diagnosis, whether patient-related or health-system related, results in worse outcomes. Our study is one of the few that has analysed and reported

the details of the break-up of delay-types and relevant timelines for each. By doing so, it is possible to work on those areas identified as causing the longest delays. Such bottlenecks once identified, frequently can be managed with minor changes in patient flow, referral systems, vetting process and so on. For example, one of the simplest to implement that could reduce delays in starting treatment would be to hand out a prescription in endoscopy for patients newly diagnosed with IBD. Individual centres will need to work on respective bottlenecks using these timelines as a target to be achieved. Timely diagnosis will very likely result in better outcomes and fewer AOs, ultimately reducing the burden on the health-system. Overall, achieving adequate control of chronic inflammation would improve short and long term outcomes for IBD patients.

Strengths and limitations of the study

Our study has several strengths. We applied very stringent criteria for inclusion to ensure only inception patients are part of the study cohort. This was done to ensure we are able to study the outcomes of sustained chronic inflammation at the start of the disease process. We have analysed delay-types in great detail and shown that specific timelines are definitely relevant to clinical outcomes. We assigned stringent but fair cut-off times to measure performance of the service, making it quite robust.

Our study has several limitations. As this was a retrospective study, there was some data missing, particularly data such as smoking status and up to date biomarkers, and it is possible that AOs may be under-reported. This data was from one tertiary care centre and the number of patients included in the study were relatively low. As our centre is a tertiary care with dedicated IBD

service, we assume our timelines may be better than other centres. Despite this advantage, even short delays appear to have an impact on clinical outcomes. Due to smaller number of patients, we were unable to perform statistically meaningful univariate or multivariate regression analysis to identify risk factors that could predict delays.

3.1.6 Conclusions

To conclude, we have shown from our data that delays, and subsequent untreated chronic inflammation leads to poor outcomes in newly diagnosed IBD patients.

Our study has shown that delays in diagnosis could be potentially detrimental. Whether it is patient-related, or health-system related, they lead to poorer outcomes. There are often several bottlenecks in a service that contribute to delays, increasing AOs in IBD over the follow-up period. In our cohort, a delay as short as a week, between GP referral and specialty review, is statistically significant in determining AOs, hence their negative impact should not be underestimated.

Each delay-type is unique in some ways and highlights a different area for improvement. Any delays by patients in seeking medical attention from the GP identifies that patients may not be aware of the potential seriousness of their GI symptoms. This could perhaps be addressed by undertaking patient education and awareness programmes locally, regionally, and also nationally. Delays in clinical appointments and endoscopy appointments can be variable based on the centres. This particular problem is pertinent during the post-pandemic recovery period as almost every hospital in the UK is struggling to clear backlogs, while simultaneously grappling with unprecedented demands placed on an already stretched workforce. This is also applicable to

specialist IBD centres where our data is likely to have implications on service delivery and planning. The data on high proportion of AOs may prove supportive in planning business cases to redesign services, add workforce and prioritise IBD patients, to minimise the damage done by excessive delays. Our data also suggests that cumulative delays in the hospital increase the use of biologics for consecutive years. This will undoubtedly add to pressures of IBD teams and infusion suites at hospitals. Specialist services will need to allocated resources appropriately to meet this demand in the long term. Both hospitals and policy makers will need to consider this and allocate necessary resources to minimise delays and keep the standards of best practice.

3.2 Discontinuation of biologic therapy in IBD

3.2.1 Background

Inflammatory Bowel Diseases (IBD) are increasing in incidence and prevalence, including in newly industrialised countries and it is now considered a global disease.(Kaplan 2015, Ng, Shi et al. 2018)

The use of biological therapies for management of patients with IBD are part of guidelines from all societies including the European Crohn's and Colitis Organisation (ECCO) as well as British Society of Gastroenterology, UK. (Lamb, Kennedy et al. 2019, Torres, Bonovas et al. 2020) The approval of drugs and guidance are based on pivotal trials (Rutgeerts, Sandborn et al. 2005, Sandborn, van Assche et al. 2012, Feagan, Rutgeerts et al. 2013, Sandborn, Feagan et al. 2014, Sandborn, Su et al. 2017, Sands, Sandborn et al. 2019) and these drugs have given clinicians multiple options to treat patients. Biologics have been a main driver of expenditure that have driven up health care costs in IBD in the past decade.(Targownik, Kaplan et al. 2020) However, real world data suggests response rates as low as 30% with a significant proportion needing to discontinue biologics due to a variety of adverse events(Juillerat, Sokol et al. 2015, Lehtola, Haapamaki et al. 2016) ; some patients choose to discontinue the drug due to personal choice (eg-pregnancy, patient perceived risk of malignancy). Further, guidelines from health economic agencies in countries such as UK suggest clinicians undertake a review of biologic therapy annually for each patient, with a view of withdrawing therapy if patients are in remission, but it does not recommend specific investigations prior to the decision.(NICE 2010) ECCO guidance on treatment withdrawal (exit strategies) has been published to help clinicians make rational cost-effective

decisions whilst managing patients, but the recommendations are based on limited evidence.(Doherty, Katsanos et al. 2018)

While such guidelines aim to rationalise therapy and ensure maintenance of economic feasibility these also bring challenges to both clinicians and patients alike. There is a real and definite risk of relapse of disease, although current evidence shows considerable variation in the proportion of patients who relapse within a year of discontinuation. As per ECCO review on biologic therapy, the risk of relapse after anti-TNF withdrawal is between 30–40% at 1 year, and greater than 50% beyond 2 years. (Doherty, Katsanos et al. 2018) This can range from 30% to as high as 60% in some studies, depending on follow-up periods. (Waugh, Garg et al. 2010, Molander, Farkkila et al. 2017, Lee, Kim et al. 2018) Based on current evidence, patients who have ‘deep’ remission confirmed objectively prior to biologic withdrawal, are more likely to remain in remission at 12 months of follow-up and this is more often seen in Ulcerative Colitis (UC) than Crohn’s Disease (CD). (Molander, Farkkila et al. 2014) To help aid the decision of withdrawal, some studies have attempted to identify risk factors for relapse after discontinuation of therapy (Lee, Kim et al. 2018) but at present there are no controlled studies to confirm the best strategy and benefits of withdrawal.(Doherty, Katsanos et al. 2018)

3.2.2 Aims

In clinical practice, patients with IBD have their biologic therapies withdrawn due to variety of reasons. The aims of the study were to -

- report outcomes of all discontinuations of biologics and predictors of prognosis, after a minimum follow-up of 24 months.

- study outcomes of patients after elective discontinuation of first-use biologics by their clinicians (after a period of clinical remission) and their direct cost implications on the health system

3.2.3 Patients and methods

All IBD patients who discontinued their biologics between January 2013 and December 2016 were identified from electronic medical records at University Hospitals Birmingham, UK which is a tertiary referral centre. This was to ensure at least 24 months follow-up for each patient, until end of December 2018.

Data on reasons for discontinuation of biologics was recorded; relevant clinical information was collected using the hospital biochemistry, radiology and endoscopy databases. A set of pre-defined adverse outcomes that included steroid & other rescue therapies, hospitalisations, surgery including perianal were recorded. We also included patients who were classed as primary or secondary non-responders to their first biologic, if they had not been switched to another biologic for a period more than 12 weeks since discontinuation. For purposes of our study, primary non-response was defined as no improvement in clinical symptoms and/or biomarkers at 12 weeks post-biologics, as recorded by the clinician; secondary loss of response was defined as relapse of clinical symptoms and/or worsening biomarkers after a period of remission on biologic therapy, as recorded by the clinician.

The outcomes of patients were recorded and analysed in two different contexts-

- as an entire cohort (elective and non-elective discontinuation) to investigate predictors of prognosis
- as a sub-group who had 'elective' discontinuation only (as decided by the treating gastroenterologist)

To assess the direct costs after discontinuation of biologic, the burden of these outcomes on the healthcare system were recorded in the form of 'episodes', during the follow-up period. Each pre-defined adverse outcome (including any form of unscheduled patient contact with the hospital) was considered an episode which required some form of action to be taken by healthcare professionals to resolve it.

Statistical analysis

The data were analysed using multivariable and univariable logistic regressions within a machine learning technique in order to predict adverse outcomes, within the stated timeframe by means of R (R Core Team (2019) package CARROT Alina Bazarova and Marko Raseta, 2019.(Bazarova A 2019) The latter combines principles of good practice from machine learning, such as cross-validation (S 1993) and those in medical statistics, such as best subset regression (Zhang 2016) restricted by the rule of ten events per variable ("one in ten rule")(Peduzzi, Concato et al. 1996). CARROT has been previously successfully used for outcome prediction in the clinical outcomes (Rutter, Crees et al. 2020, Smith, Bazarova et al. 2020) We tested the significance of the identified predictors and performed Kaplan-Meier (Kaplan 1958) and Cox proportional hazards (Cox 1972) survival analysis to compare patients with elective versus non-elective discontinuation of biologics. The patients who had their biologics electively discontinued were analysed again as a

separate group to detect any differences in their outcomes. Survival analysis and likelihood-ratio test to assess the separation between survival curves were performed by R package survival and visualised via the package R survminer (Kassambara A 2019).

Ethical considerations

This study was registered with hospital research governance & ethics committee and was given approval to proceed in April 2019. (*Reference number CARMS-15164*)

3.2.4 Results

147 patients who discontinued biologics (M=74, median age 39y; CD=110) were identified. Follow-up ranged from 24-60 months (median 40 months). The details of demographics and patient characteristics are given in **Table 3-5**.

Table 3-5 Demographics of patient characteristics n=147

Age & gender	N=147
Median age	39 years (range 21-82 years)
Gender	Male=74; Female=73
Disease type	
Ulcerative colitis	37 (25.2%)
Crohn's disease	110 (74.8%)
Race	
Caucasian	102 (69.4%)
Asian	23 (15.6%)
Afro-Caribbean	4 (2.7%)

Unknown/Unreported	18 (11.9%)
Montreal classification for CD	
Age	
A1	37
A2	70
A3	0
Unknown	3
Location	
L1	24
L2	34
L3	39
L4	12
Unknown	5
Behaviour	
B1	3
B2	26
B3	17
Perianal	23
Unknown	41
Ulcerative colitis extent	
E1	1
E2	9
E3	18
Unknown	9
Follow up period after discontinuation	
24 months	147 (100%)
>24 months (median 40 months; range 24-60 months))	80 (54.5%)

The largest number of patients [59 (40%)] had their therapy discontinued electively, which was recorded as a joint decision by the treating clinician and patient. This decision was based on

assessment of clinical remission using global assessment and blood markers of activity of disease (CRP, full blood counts). In our cohort we noted that endoscopy, cross-sectional imaging or histology were used at the discretion of the clinician but not routinely used before discontinuation of the drug. The common reasons for non-elective discontinuation were drug intolerance (14%), secondary loss of response (12%), primary non-response (10%) and patient choice (7%) among others.

Elective versus non-elective discontinuation of biologics

Among patients who had their biologics discontinued, 60% of patients were non-elective. The most common reasons in this group of patients were side effects (not otherwise specified), primary non-response or secondary loss of response, patient choice, immune reactions to the drug and patient non-adherence. All patients had their outcomes recorded at pre-specified time frames, with a minimum of 24 months follow-up. Using data from all 147 patients, multivariable logistic regression analysis was performed to identify significant predictors of poor and favourable outcomes. These are represented in **Table 3-6**.

In this entire cohort, elective discontinuation resulted in fewer IBD related pre-defined adverse outcomes compared to non-elective ($p=0.003$). **Figure 3-10** shows a Cox Proportional Hazards curves comparing the two cohorts.

Although there is good separation between the two groups initially, there were a significant proportion of patients with adverse outcomes within 6 months of biologic discontinuation in both

groups and the curves began to merge at about 40 months which was the median duration of follow up in this study.

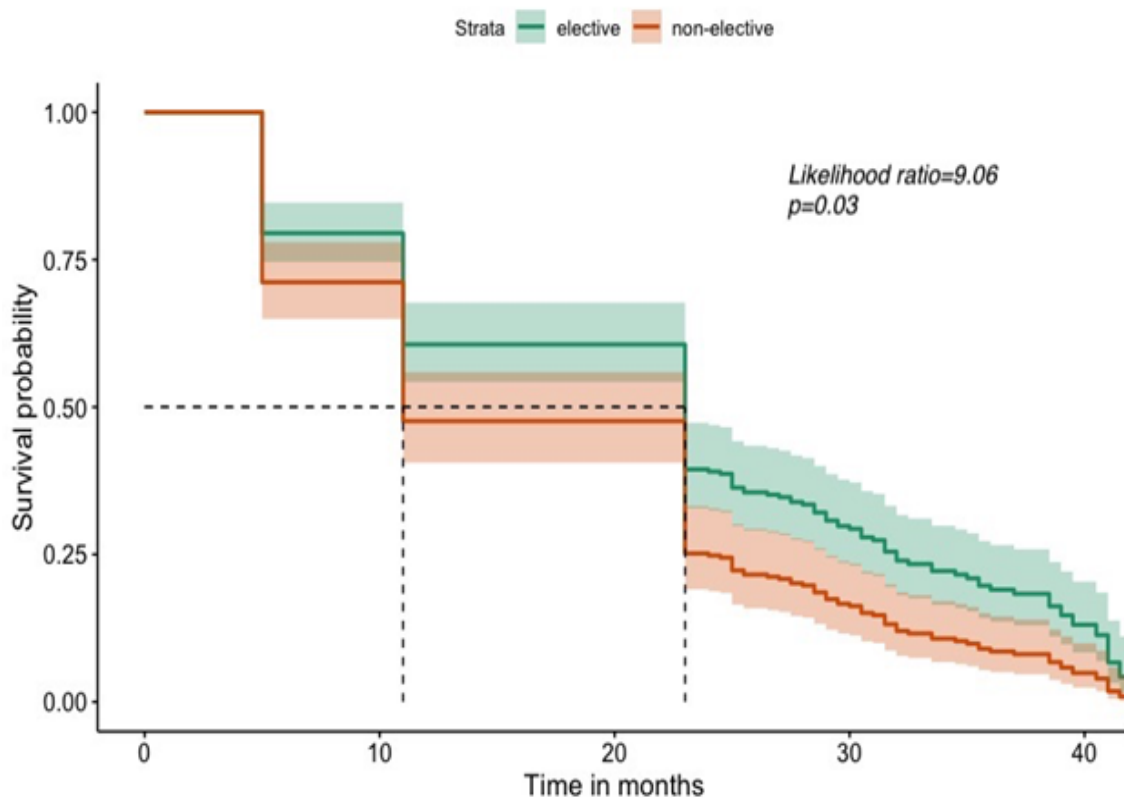


Figure 3-10 Survival without pre-defined adverse outcomes (elective vs non-elective discontinuation)

Table 3-6 Predictors of outcomes in entire cohort

Predictors of poor outcomes	Time interval when significant	AUROC	Odds ratio (OR) and confidence intervals	Statistically significant?
Asian race	< 6months	0.6283	OR 0.68 95% C-I (0.49,0.95)	Yes
Secondary non-response	< 6months 6-12months 12-24months >24 months	0.6715	OR 7.09 95% C-I (1.60,29.97)	Yes
Steroid therapy following discontinuation	12-24 months	0.6219	OR 0.24 95% C-I (0.04,0.88)	Yes
Predictors of good outcomes				
Male sex	>24 months	0.638	OR 0.31 95% C-I (0.09,0.85)	Yes
Elective stop	<6 months 6-12 months	0.6398	OR 0.24 95% C-I (0.05,0.77)	Yes

In the total cohort, significant number of patients (n=80, 54%) had adverse outcomes within 6 months of discontinuation, and 96 (65%) patients needed biologics to be restarted by the end of study follow-up period.

Elective discontinuation of first-use biologic

Of the total cohort, 59 patients had their therapy discontinued electively; median duration of therapy prior to discontinuation was 24 months (range 1m-96m). This group was analysed separately to better understand outcomes. The decision to discontinue biologics was made by the treating clinician after discussion with the patient, keeping in line with national guidelines provided by NICE, UK. Further clinical details were collected for these 59 patients, which are as given in **Table 3-7**.

Among these patients, all had been on anti-TNFs; 44(75%) had received therapy for more than 12 months with no differences noted between infliximab and adalimumab and therefore considered together.

26(44%) patients continued thiopurines after biologic stoppage. Patients not on thiopurines had a trend towards lower probability of adverse outcomes during follow-up period, however, the difference between two groups of patients was not statistically significant ($p=0.32$, likelihood ratio=0.98) (**Figure 3-11**).

Table 3-7 Details of patients with elective discontinuation of biologics (n=59)

Type of first-use biologic	59/59
Adalimumab	32
Infliximab	27
Disease classification	
Ulcerative Colitis	9
Crohn's Disease	50
Montreal classification for CD	
Age	
A1	18
A2	27
A3	5
Unknown	0
Location	
L1	10
L2	17
L3	22
L4	7
Unknown	0
Behaviour	
B1	1
B2	14

B3	8
Perianal	8
Unknown	29
Ulcerative colitis extent	
E1	0
E2	2
E3	7
Duration of biologic therapy prior to stop	59/59
< 6 months	9
6 to 12 months	6
13 to 24 months	16
25 to 36 months	11
> 36 months	17
Thiopurine continued after biologic stop	59/59
Yes	26
No	33
Patients needing restart of biologic therapy (44/59)	44/59
< 6 months	9
6 to 12 months	13
13 to 24 months	10
> 24 months	12

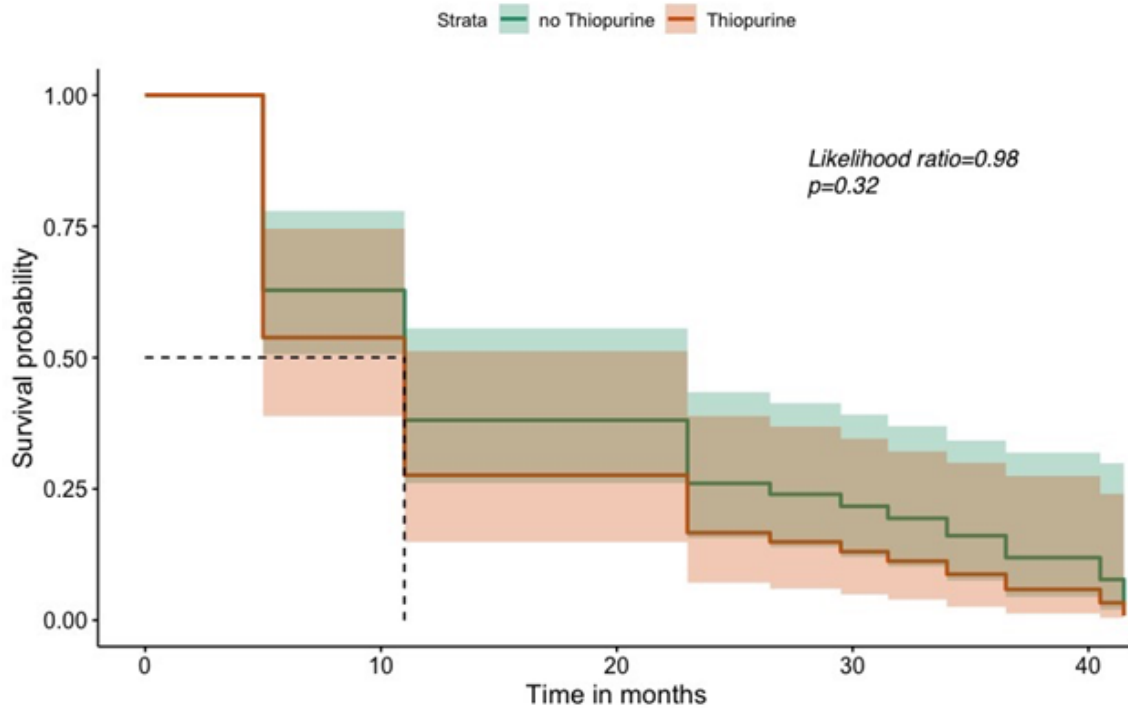


Figure 3-11 Survival without any pre-defined adverse outcomes in thiopurine vs no thiopurine

In this sub-group, 23 (40%) patients had IBD related adverse outcomes within 6 months after stoppage and 53 (90%) patients had at least one adverse outcome recorded by the end of follow-up. It was also noted that 44 (75%) patients needed to restart biologics at some point during the follow-up period of this study. The proportion of patients without any adverse outcomes after elective discontinuation is demonstrated in using a Kaplan-Meier survival curve. **(Figure 3-13)** More than 50% of patients had to restart biological drugs within 2 years of discontinuation.

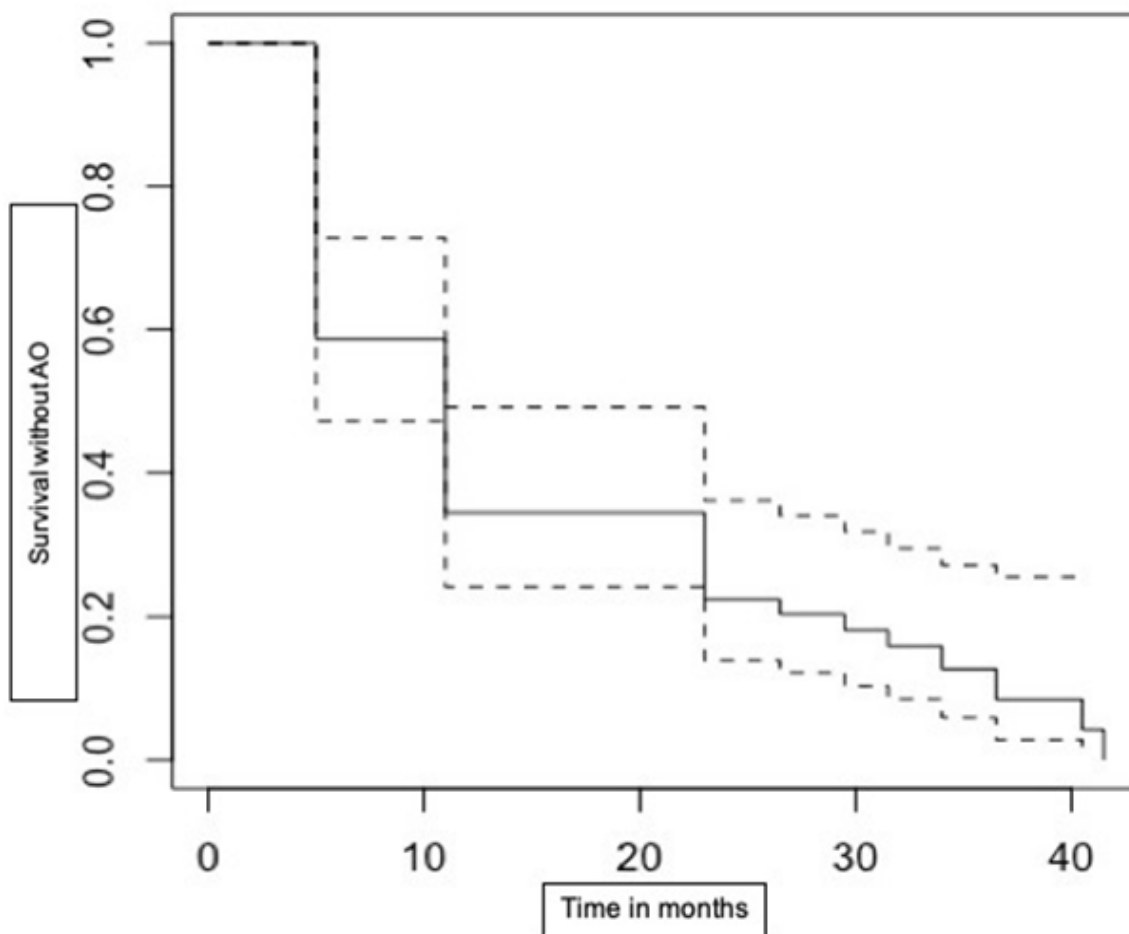


Figure 3-12 Survival without any pre-defined adverse outcomes among elective discontinuations

AO- adverse outcomes

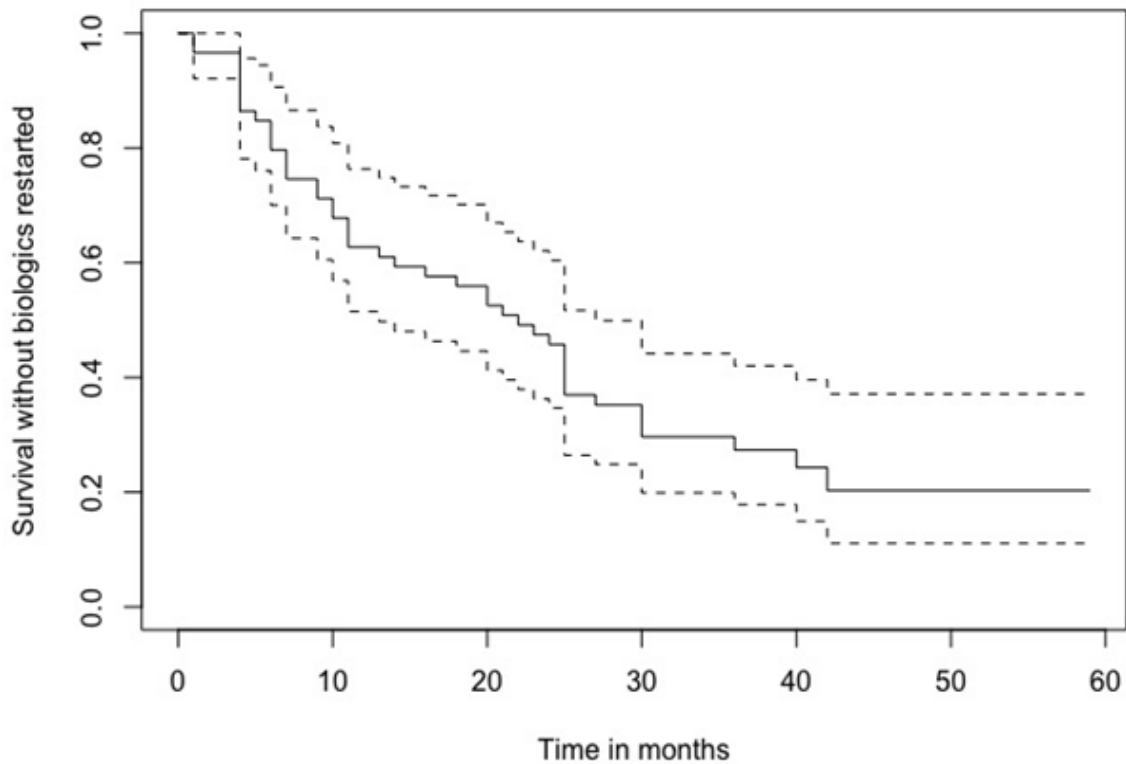


Figure 3-13 Survival without biologic restart among elective discontinuations

Episodes of contact with healthcare team

In this cohort of patients, we also studied the number of IBD related unscheduled contacts (triggered by adverse outcomes) these patients had with the healthcare team following elective discontinuation of biologics. Each contact was termed as an ‘episode’ and data was collected for pre-specified time frames. Each patient could have had more than one episode during the follow-

up period, and this was collected as cumulative data. The pre-defined adverse outcomes were flare-up, corticosteroid prescription, hospitalisation, rescue therapy (using urgent biologic therapy as inpatient or urgent ambulatory care) and surgery (including procedures for perianal disease).

Consequently, each patient could have had multiple episodes over the follow up period. This data was collected in a cumulative fashion to assess the economic burden on the healthcare system as a result of these multiple episodes. The costing was calculated using data from 'unit pricing' at a national level, and service & drug costs at our centre.

For purposes of the study and to calculate financial burden, we made the following assumptions

- all episodes which followed discontinuation of biologics were a consequence of the discontinuation itself
- all of the episodes could have been avoided had the biologic been continued

While these are assumptions, these allow calculations which are likely to be a close reflection of real-world experience of most teams caring for patients with IBD.

The breakdown of the number of episodes at pre-specified time points and the cumulative total is given in **Table 3-8**. In our cohort, it was noted that an episode of contact recorded due to a flare-up was the commonest followed by rescue therapy with biologics. The number of episodes varied with time points but noted to increase beyond 24 months of follow-up.

Table 3-8 Details of episodes at pre-specified time points and cumulative total

Adverse outcomes (‘Episodes’)	< 6 months	6-12 months	12-24 months	> 24 months	TOTAL for EACH AO
Flare-up	20	18	20	24	82
Corticosteroid therapy	6	5	5	5	21
Rescue therapy (using biologics)	10	14	14	20	58
Hospitalisation	1	0	1	5	7
Progress of disease (Stricturing, penetrating or perianal complication)	3	3	5	5	16
Surgery	2	0	3	3	8
TOTAL EPISODES as per time frame	42	40	48	62	

The estimated economic burden of episodes following discontinuation of biologics was calculated using the National Health Services (NHS) UK unit-price costs for health and social care list for 2017.(Curtis 2017) A similar approach has been reported by previous studies that have investigated the economic aspects.(Travis, Feagan et al. 2017)

A pragmatic approach was used to calculate the direct costs. In our centre, flare-up of disease generally triggers a telephone call by the patient to the dedicated IBD helpline for advice. The unit cost is calculated using the costing for a telephone clinic. The total cumulative cost for all elective discontinuations by type of episode is given in **Table 3-9**.

Table 3-9 Details of estimated immediate direct costs following elective discontinuation

Episode	Services utilised	Unit price in GBP (£)	Total number of episodes	Total cumulative cost
Flare-up	Telephone call/clinic	25	82	2050
Corticosteroid therapy	Prescription	9	21	189
	Outpatient attendance	137	11	1507
Rescue therapy using biologics	Outpatient attendance	137	58	7946
	Multi-disciplinary team meeting	243	58	14094
	Cost of drug/s			
	Infliximab*	1200	66 infusions (11 pts)	79200
	Adalimumab**	140	754 injections (29 pts)	105560
	Ustekinumab***	2150	24 injections (4 pts)	51600
	Day-case unit for infusion (only for IV drugs)	300	66	19800
Hospitalisation	Inpatient stay	727	7	5089
Surgery including hospital stay		3900	8	31200
GRAND TOTAL				266635

*Calculated for 12 months at 8 weekly infusions (presumed avg dose of 300mg/dose)

**Calculated for 12 months at fortnightly injections (presumed avg of 40mg/dose)

***Calculated for 12 months at 8 weekly injections (avg dose of 90mg; induction dose excluded)

3.2.5 Discussion

Biologics are routinely used in IBD. With a large number of IBD patients now on biologics, they account for a major proportion of costs incurred and hence the expenditure in health systems for management of IBD is now driven by medical therapies which are expensive.(Targownik, Benchimol et al. 2019) As a result, health care agencies such as the National Institute for Health and Care Excellence (NICE) in UK provide guidance to clinicians to rationalise therapies.(NICE 2010) One of the ways this is done is by reviewing need for biologic therapy every 12 months and encouraging elective discontinuation in patients who have achieved disease remission. No specific standards to define remission are recommended. While this is done keeping economic feasibility in mind, it is debatable whether the strategy does in fact pay dividends. Although expensive, it has been shown that among patients who respond to biologics, a significant proportion remain in sustained remission for up to 5 years (Eshuis, Peters et al. 2013, Peters, Eshuis et al. 2014), with a reduction in need for surgery and hospitalisation(van der Valk, Mangen et al. 2014), and over years, the direct expenditure on the health system reduces.(Burisch, Vardi et al. 2020) Despite the high perceived costs it could be argued that these drugs bring net benefits and should be continued without forced elective interruptions. The rates of relapse and resulting need for high intensity care in patients who have had their drugs discontinued probably offset the costs saved by elective discontinuation. This is especially true given the changing landscape with introduction of biosimilar drugs driving down cost of medications.

Apart from elective discontinuation, there is a proportion of patients in whom treatment has to be discontinued for reasons that have been discussed before. In patients with primary non-

response, secondary loss of response or drug intolerance, the treatment is usually switched to another approved agent. However, despite the best efforts of most teams managing IBD patients, it is seldom possible to ensure that the switch is done in a timely manner. There could be multiple reasons that lead to a delay e.g.- delays due to patient related factors, delays in the system, lack of therapeutic options on offer and many more. Regardless of reasons for the delay, it could be argued that delays have the same detrimental effect as discontinuations, invariably leading to higher rate of complications.

Predictors of poor and favourable outcomes after discontinuation

The outcomes after discontinuation have been reported by other studies previously, most of which have been assessed in patients who have objective confirmation of remission prior to discontinuation. As per a systematic review by Torres et al, the rates of relapse after discontinuation range from 20% to as high as 80%, particularly in some high-risk patients.(Torres, Boyapati et al. 2015)

In our total cohort, a larger proportion of patients had non-elective discontinuation of therapy. The predictors of poor outcomes were Asian race, secondary loss of response (at any time point) and need for steroid therapy at 12 months or more after discontinuation, all of which reached statistical significance. **(Table 3-6)** The poor outcomes due to secondary loss of response is perhaps not unexpected and could represent refractory or aggressive disease. This highlights the group of patients who need closer monitoring and a more proactive management plan. This is also true of patients requiring steroid therapy and such patients should be considered for a swift change to a different biologic or other interventions as appropriate.

The analysis also showed male sex and elective discontinuation as predictors of good outcomes, reaching statistical significance. Although patients who have elective discontinuation have been shown to have slightly better outcomes (Casanova, Chaparro et al. 2017), male patients doing better after discontinuation has so far not been reported commonly. It is possible that elective discontinuations do better mainly because the patients are more likely to have achieved clinical remission and also more likely to have biomarker assessments undertaken prior to withdrawal of therapy.

There have been reports on different aspects of discontinuation (including patients at risk of discontinuation itself) and several investigators have attempted to recognise predictors of outcomes. The STORI study stratified patients and identified those at low risk of relapse using predictive factors.(Louis, Mary et al. 2012) A recent study published in 2019 reported that female sex was associated with a higher risk of discontinuation of anti-TNF therapy in IBD due to higher rates of drug intolerance.(Schultheiss, Brand et al. 2019) In the same study, other factors such as greater age at start of anti-TNF therapy and dose escalation were found to be associated with discontinuation.(Schultheiss, Brand et al. 2019) Similar findings were reported by another study where a greater age of patient was associated with higher risk of discontinuation and treatment failure.(de Jong, Smits et al. 2020) Our cohort identified some new risk factors which predict poor outcomes after discontinuation of biologics which could guide decision making.

Elective discontinuation of anti-TNF therapy

In our centre, patients were assessed for clinical remission based on symptoms and serum biomarkers prior to discontinuation of therapy. Mucosal assessment by way of endoscopy was not always undertaken and faecal calprotectin was available only after 2017, hence not used in this cohort. Among the elective discontinuations in our cohort we made some interesting observations. Patients who continued to remain on thiopurine therapy after withdrawal of biologic had more adverse outcomes recorded during follow-up period. **(Figure 3-11)** This is contrary to most reports which show thiopurines to have a protective effect, particularly in CD. (Casanova, Chaparro et al. 2017)

The cause for concern is the large number of patients who have adverse outcomes within a very short duration after discontinuation of therapy. About 40% of patients had at least one or more adverse outcome within 6 months of discontinuation and this number steadily increased with time, with nearly 90% of patients having at least one adverse outcome at the end of follow-up period. **(Figure 3-12)** Also, a very high proportion of patients (71%) required restart of biologics during the follow-up period to manage adverse outcomes. **(Figure 3-13)** The relapse rate in our cohort was much higher than reports in most studies, including a systematic review by Gisbert et al. (Gisbert, Marin et al. 2016)

It is unclear whether these patients had relapsed due to underlying disease activity which was asymptomatic and not detected on serum biomarkers. It is now well established that patients who achieve mucosal healing are more likely to remain in remission for longer durations as well as have lower rates of hospitalisation and surgery (Schnitzler, Fidler et al. 2009, Baert, Moortgat et al. 2010, Pineton de Chambrun, Peyrin-Biroulet et al. 2010); it is also recommended that

treatment should be withdrawn only after objective confirmation of mucosal healing.(Doherty, Katsanos et al. 2018) The significantly high number of patients with adverse outcomes in this cohort emphasises the importance of objective confirmation of mucosal healing in order to reduce rates of relapse.

Cost of treatment withdrawal/discontinuation

The number of episodes where patients came in contact with the healthcare team following elective discontinuation brings cost implications. A calculation of cumulative direct costs for all patients showed a high expenditure in managing these episodes. These episodes also increase time pressures on teams as they consume valuable resources. **(Table 3-9)** The indirect costs due to patient morbidity are probably higher but this was not explored as it was outside the scope of this study. A very high proportion of patients also needed to restart biologics to manage relapses. Any theoretical economic benefits gained by discontinuation of therapy are offset by costs incurred in managing the adverse outcomes. These episodes and resulting costs probably outweigh the theoretical benefits of discontinuation. This is especially more relevant considering the introduction of biosimilars which are available at much lower prices as well as the significant reduction in the cost of the originator drugs due to discounted pricing. The annual cost of maintaining patients on these drugs is now significantly less. The lower cost has to be capitalised on in the form of maintaining therapies to maintain remission for as long as the drug remains effective. The guidelines by health agencies to review use of biologics annually were partially driven by the high cost. However, since the approval of biosimilar agents for both infliximab and adalimumab, the health services are projected to make large savings.(England 2018) The end of

patent as well as introduction of biosimilars has resulted in pharmaceutical companies offering heavy discounts of up to 70% on originator drugs, resulting in further reduction of costs.(Biopharmadive 2015) Biosimilars have been reported to be as safe and effective as originators.(White 2016) Hence, there has been a drastic overall reduction in the annual cost per patient in managing diseases without any compromise in efficacy. This brings into question whether treatment withdrawal in patients who have achieved remission is a sound strategy. The area of treatment withdrawal or de-escalation is poorly understood and continues to evolve. There are studies from different regions which show variation in outcomes after withdrawal. Some studies from South-Asia show better outcomes at 24 months following discontinuation of biologic. (Banerjee, Pal et al. 2021) ECCO guidelines however do not recommend treatment withdrawal in high-risk patients (perianal disease, penetrating disease) but given the lowering cost and comparable safety profile of anti-TNF monotherapy to thiopurines, this should probably be extended to most patients who have been successfully and safely maintained in remission.

3.2.6 Limitations of study

Our study has several limitations. As this was a retrospective study, there was some data missing, particularly data such as smoking status and faecal calprotectin levels. Another limitation of our cohort, which probably reflects real-world practice at the time is that even when biologics were discontinued electively, the decision was not always after confirming endoscopic or histological remission. This was more often based on clinical remission as identified by the clinician together with blood and serum biomarkers. This approach is unlikely to have been a reliable representation of true disease activity, but pressure on endoscopy services made ready access often challenging.

About 22% of patients faced significant delays in switching to alternate biologic therapies after primary non-response or secondary loss of response was identified, due to various reasons, and this delay probably had an effect on frequency of adverse outcomes. Therapeutic drug-level monitoring was not available for routine care at the time of the study.

To assess economic aspects the calculations of cost were based on a pragmatic approach where we assumed that a particular episode of adverse outcome would generally trigger certain services in the health system. It is quite possible that the estimates could be an underestimate as it was difficult to track how each episode was resolved. Another limitation is the challenge in assessing direct costs of progression of disease or its behaviour. When patients have penetrating or perianal complications, multiple services are inevitably utilised, but it may not always be possible to capture in retrospective studies.

3.2.7 Conclusions

The discontinuation of biologics for variety of reasons is a common clinical scenario in real-world practice. In our cohort, among patients who discontinued biologics, nearly 54% had at least one adverse outcome within 6 months of discontinuation. Our results show that there were fewer adverse outcomes when they were electively discontinued, even when disease assessment prior to discontinuation was based on clinical symptoms and serum biomarkers alone. However, despite elective discontinuation, a large proportion had adverse outcomes rapidly and about 75% required restart of biologics. These adverse outcomes resulted in an increased number of episodes of contact with the healthcare team, thereby resulting in added expenditure in managing them.

Clinicians need to be cautious when considering biologic discontinuation (including electively) given the high proportion who relapse and need reescalation to biologics. This should be discussed with patients when considering discontinuation of biologics. It can be argued that the direct costs of increased frequency of contacts with the healthcare system offset the economic gains made by drug withdrawal. This is especially true considering the reducing costs of drugs and increasing use of biosimilars. Clinicians and health agencies should take cognisance of the changing economic equations in making decisions and policies.

4 CHAPTER 4: TISSUE REMODELLING AND FIBROSTENOSIS IN CROHN'S DISEASE-STUDY OF MOLECULAR SIGNALS AND BIOMARKERS (TRAFIC STUDY)

Parts of this chapter have been presented at ECCO, DDW and UEG conferences

Akiror S, Acharjee A, Jeffery L, Shivaji, UN et al.

Sa132 Transcriptome analysis identifies dysregulated pathways and targets for therapy in patients undergoing surgical resection for Crohn's disease. (Akiror, Acharjee et al. 2021)

Gastroenterology. 2021;160(6):S-432.

4.1 Background

In the retrospective study (chapter 2), it was noted that chronic inflammation is a predominant component of intestinal strictures resected at surgery despite the pre-surgical assessment of these strictures as 'fibrotic'. Our results show that 'pure' fibrosis is unusual and chronic inflammation is constantly present in all strictures, along with muscular hyperplasia, in all layers of the bowel wall. Although it is generally accepted that inflammation is likely to be one of the main drivers of fibrosis, our retrospective study provides evidence of this at a histological level. This finding would suggest that some of the pathways and mechanisms responsible for a sustained inflammatory state probably promote or trigger changes that lead to fibrosis.

It is very likely that fibroblasts, myofibroblasts and smooth muscle cells contribute to fibrosis & volume expansion in a stricture. The process is promoted by pro-fibrotic mediators (Rieder, Zimmermann et al. 2013) and opposed by anti-fibrotic mediators. The loss of equilibrium between pro & anti-fibrotic mediators is probably lost at some point leading to intestinal fibrosis.

Inflammation and fibrosis are closely related. There is likely to be an interplay between the two and it has been recognised as probably one of the main drivers of fibrosis in not only the gut but multiple organ systems. It has also been noted that in patients with CD there was improvement in strictures or delay information of strictures by effective control of inflammation with biologics and immunomodulator therapy.(Safroneeva, Vavricka et al. 2015)

It has been proposed that some patients with a non-inflamed bowel (based on clinical parameters), also go on to develop fibrosis. It has been noted that the extracellular matrix (ECM) laid down by activated mesenchymal cells acts as a storage site for cytokines and fibrotic growth factors, including TGF beta. The release of these promotes deposition and crosslinking of ECM

components which could change its local mechanical properties this making it stiffer. The stiffness by itself could further stimulate fibrogenesis by activating mesenchymal cells, even in the absence of other activating factors.(Rieder, Fiocchi et al. 2017) The mesenchymal cells could further increase stiffness independent of inflammation.

4.2 Fibrosis in CD and stricturing disease

The most common presentation of CD is inflammatory; about 50% of patients will develop stricturing or penetrating complications in the first 10 years after diagnosis.(Louis, Collard et al. 2001, Cosnes, Cattan et al. 2002, Cosnes, Gowerrousseau et al. 2011) Stricture formation is a result of intestinal fibrosis and can occur at any time and involve any intestinal segment, the commonest site for stricture being the terminal ileum and ileo-colonic region.(Peyrin-Biroulet, Loftus et al. 2010) The stricturing phenotype (B2) of the CD eventually and frequently requires surgery to manage the symptoms (mainly pain) and complications (e.g. intestinal obstruction). Although the overall risk of intestinal surgery among patients with IBD has decreased over the past six decades, the risk remained significantly high (16.3%, 33.3%, and 46.6% at 1, 5, and 10 years, respectively, after diagnosis of CD).(Frolkis, Dykeman et al. 2013)

4.3 Management strategies for CD related strictures

The management of strictures includes medical therapy and interventional therapy. Medical therapy is mainly in the form of drugs to control inflammation. There are no anti fibrotic agents available to treat intestinal fibrosis. Biological therapy is the most common in this phenotype. It

is either used as monotherapy or in combination with an immunomodulator. Biologics have been shown to slow the progression or in some cases even regress the strictures.(Bouguen G 2009, Pelletier AL1 2009) Inflammatory strictures can be managed using measures to reduce disease activity but fibrotic strictures can pose a challenge.(Holtmann, Wanitschke et al. 2003, Echarri A 2011). They are unlikely to respond to medical therapy and therefore endoscopic intervention or surgery are the next available options.

Interventions include endoscopic dilatation and surgical intervention like stricturoplasty or resection of the affected segment. However, recurrence is seen in nearly a third of patients after surgery.(Bharadwaj and Shen 2016) Further details of surgical techniques are discussed in section 1.2.4.

Overall, the management of strictures remains challenging, and outcomes could probably be termed as sub-optimal. There has been some success reported with use of biologics. It has also been noted that regular therapy with biologics increases likelihood of clinical remission, mucosal healing with a reduction in the need for surgery and hospitalization, in both clinical trials and clinical practice.(Mandel MD1 2014) Fu et al noted that biologics have increased the median time to surgery; however, in their cohort interestingly there was a shift from inflammatory to stricturing & penetrating phenotypes.(Fu, Hong et al. 2014)

It has been a massive challenge to develop anti fibrotic treatments to effectively treat intestinal strictures. Although there are some anti fibrotic drugs tested and approved in other organs systems, no effective drug has been found to decelerate, treat, or regress intestinal fibrosis. It remains a massive unmet need and an area requiring more research. With the knowledge from our previous data that sustained CI at the level of the tissue probably drives fibrosis, we wanted to further study the molecular aspects of a stricture and compare with non-strictured bowel.

These molecular biomarkers may reveal some novel pathways that are relevant in sustained CI and fibrosis, which then could be used as potential targets of therapy.

We planned a prospective study designed to investigate the inflammation-fibrosis interplay detail using multiomics techniques to contribute to this area where there is an unmet need.

4.4 Hypothesis and study objectives

After extensive discussions with my supervisors Professor Subrata Ghosh and Professor Marietta Iacucci about the results of the three retrospective studies, a prospective study was planned to investigate multiple aspects of IBD. The main areas of interest were chronic inflammation and resulting fibrosis in intestinal segments. We discussed the hypothesis and study objectives.

The hypothesis was based on the findings from our previous results. Chronic inflammation results in multiple long term adverse outcomes (AOs). It is likely that CD patients undergoing surgery for non-inflamed fibrotic disease may in fact have active chronic inflammation at the tissue and stricture microenvironment level, which may not be apparent on routine clinical or biochemical testing. The study of strictures, proximal and distal margins of resected tissue may provide some insights into inflammation at a molecular level. The application of multiomics could give us more insights into activated pathways, state of inflammation and novel targets. The study acronym we used was TRAFIC (Tissue remodelling and Fibrostenosis in Crohn's Disease) – a study of biomarkers and molecular signals. It was popularly known in our group as the TRAFIC study.

The study objectives we outlined were as follows-

Primary objective

- Molecular and biomarker studies (multiomics) of surgically resected bowel to better understand the mechanism of inflammation and fibrosis in CD

Secondary objectives

- Study transcription factors and biomarkers in fibrotic segments and margins of resected bowel
- Study histology and immunohistochemistry of strictures in detail and correlate to disease course
- To compare findings among various cohorts which will serve as comparators
- To assess for any objective evidence of reversal of fibrosis (endoscopic, radiological) with recession of biomarkers in patients who respond well to biologics
- Study potential targets of therapy in vitro for anti-inflammatory and anti-fibrotic properties

4.5 Study set up

4.5.1 Steps taken prior to study approval

The study set up was an excellent, enriching and fulfilling experience for me. This was a large collaborative effort involving a very large team of research coordinators, senior clinical research fellows (PhD PGRs), laboratory research fellows (PhD PGRs), post-doctoral scientists and senior research nurses. The details of each of the team members is given in **Appendix 2**.

The initial study protocol was written by me over about 4 weeks. I also designed the Patient Information Sheet (PIS) and the Informed Consent Form (ICF) for the study. These documents went through several drafts and included inputs from my supervisors and all team members for relevant sections. We also included the Patient and Public Engagement service at the University of Birmingham (UoB) to guide us on certain aspects of the study. As part of the set up process, we held multiple discussions (Ms Laura Chapman, UoB) and a Patient Engagement meeting prior to finalising the protocol. We got very valuable inputs about the research questions, methodology and objectives of the study from patients with IBD and lay people who are interested in medical research. The final approved versions of the PIS and ICF are in **Appendix 2**.

4.5.2 Patient & Public Engagement

We followed best practice and recommendations by UoB for patient engagement in research. Patient and public opinion, inputs and recommendations were received during multiple interactions. The first interaction was during the Biomedical Research Centre (BRC) showcase held at University Hospitals Birmingham in May 2018. This event showcased planned research projects and displayed relevant apparatus. The purpose was to interact with patient focus groups and patient representatives to seek suggestions, views, opinions, and feedback about upcoming and ongoing research projects. This is a dynamic process and inputs are taken during a study period also to make improvements.

Once this was done, the suggestions were incorporated, and the final version of the protocol was prepared. The final version was ratified by our team members after about 6 weeks, and we went to the next stage of study set up.

4.5.3 Study approval

The protocol was designed to recruit patients for the collective objectives of the study. After multiple discussions the final version of protocol was submitted using the IRAS portal for consideration and approval by the Research Ethics Committee. (REC)

As per the protocol, the range of work was described clearly. It included clinical data for analysis as well as extensive wet-lab work to explore new biomarkers and study the role of fibroblasts+myofibroblasts in sustaining inflammation in the stricture microenvironment. The study included many stems which were implemented by different team members.

One of my projects within this large umbrella study was a collaborative effort with my colleague Sarah Akiror (Dr), and we worked closely to achieve our objectives. As a result, small parts of the output on which we collaborated are shared between our respective theses. The other parts of my work included ex-vivo work on drug repurposing in IBD. We had overall training and supervision for lab work on both these projects by Dr. Louisa Jeffery, a senior post-doctoral scientist in the team.

Once the study was submitted to the REC, we had a favourable outcome rapidly from the REC. Following this we got approval from the HRA, Research Governance at UoB and R&D at University Hospitals Birmingham. [REC reference number 19/SW/0010 and Study registration number RRK 6508]

4.5.4 Study protocol

The study was designed as a longitudinal, descriptive, non-interventional prospective cohort study to explore novel biomarkers associated with course of disease.

The relevant parts of the protocol and recruitment pathways proposed to include patients in the study are given below. The protocol underwent minor amendments over the next 6 months, based on our experiences and interim meetings with the patient focus group.

The background and rationale for the set-up of this large study has already been discussed. The parts of the protocol included here are the inclusion and exclusion criteria, samples to be obtained and process of approaching the patients. As this is a comprehensive protocol, the other sections are included in Appendix 2.

As per our study design, we planned to collect surgical resection tissue after IBD surgery, intestinal biopsies during endoscopy and blood samples from patients and controls.

Eligibility criteria

Eligibility criteria for surgical resection tissue

Inclusion criteria

- Able to provide informed consent
- Aged ≥ 18 and ≤ 80 years
- Confirmed diagnosis of IBD at least 1 month prior to surgery
- Patients who have been advised to have surgery to help alleviate symptoms

Exclusion criteria

- Presence of terminal ileal phlegmon with sepsis or pelvic abscess causing sepsis
- Patients with latent tuberculosis or suspected abdominal tuberculosis
- Patients undergoing emergency surgery where diagnosis of IBD is not clear or established.

Eligibility criteria for blood samples

Inclusion criteria

- Patients able to provide informed consent
- Patients aged ≥ 18 and ≤ 80 years
- Patients with confirmed diagnosis of IBD OR patients referred to Gastroenterology to investigate symptoms (non-IBD control group)

Exclusion criteria

- Severe anaemia with haemoglobin $< 80\text{g/L}$ on previous blood tests within the last 6 months

Eligibility criteria for intestinal biopsies

Inclusion criteria

- Able to provide informed consent
- Aged ≥ 18 and ≤ 80 years
- Confirmed diagnosis of IBD
- Patients referred to Gastroenterology to investigate symptoms (non-IBD control group)

Exclusion criteria

- Contraindication for colonoscopy and/or biopsy

Consent process (as written in the protocol)

Patients identified as eligible, and who have agreed to be approached, will be contacted by a member of the research team at their clinic appointment, by post or on the surgical admission ward. Patients will be given a copy of the patient information sheet (PIS) and opportunity to discuss the study. Written informed consent will be obtained from those patients willing to take

part. If a patient has already given consent to the study and then loses mental capacity, the patient will be withdrawn from the study and no further samples will be taken for research purposes. However, results of the samples that have already been taken and processed will be used as part of the study.

Sample Collection (as written in the protocol)

Once collected, surgical resection tissue, biopsies, saliva, and blood samples will be given a unique reference number to protect patient identity. The UK site will send samples to research laboratory(ies) at the University of Birmingham. Full instructions will be provided to the site.

A Material Transfer Agreement (MTA) will be in place.

Samples from patients recruited by Indian sites will be collected and analysed in India.

Surgical resection / full thickness tissue (as written in the protocol)

Tissue resected during surgery will be transferred to the histopathology department. Those patients undergoing surgery are having this intervention as part of their standard care treatment. Further samples from the resection tissue will only be taken from the surplus tissue after the intestine has been fully removed surgically from the patient. To clarify, the bowel tissue required for research is taken only after the affected part of the bowel has been removed. This is surplus tissue from which pathologists take relevant samples and then discard the rest as biological waste. Surplus tissue samples from areas of interest within the resected sample are collected for research purposes. The amount of tissue taken for research is as deemed appropriate by the pathologist. Samples obtained are then sent to the University of Birmingham laboratories.

Blood (as written in the protocol)

After patients have consented to be part of the study, blood samples will be collected in EDTA bottles for research. The blood samples that will be taken for research will be additional to what is taken for standard care and not left-over samples. Therefore, where they are in addition to standard care bloods, the total volume of blood at one sitting will not exceed 50mls (approximately 25mls to 30mls for research). The blood samples obtained are sent to University of Birmingham laboratories

Biopsies (as written in the protocol)

After patients have consented to be part of the study, biopsies will be taken for research purposes in addition to standard care biopsies during the endoscopy procedure. The biopsy samples that will be taken for research will be additional to what is taken for standard care and not left-over samples. The number of biopsies will be within safe limits as deemed appropriate by the clinician. If biopsies for standard care are not necessary, only research biopsies will be taken. The biopsies obtained for research purposes will sent to UoB laboratories. All patients attending endoscopy for standard care have a wide range of conditions, which cannot all be covered in this protocol. The number of biopsies taken is at the discretion of the endoscopist which is based on many factors for example number needed for standard care, anticoagulation use and bleeding risk, time pressures in the list, risk assessment and so on. Taking biopsies is generally safe and they are usually each 2-4mm in size. For example, as part of standard care in surveillance, a total of up to 40 biopsies are taken safely although we are moving away from this practice for different reasons. The proposed pathway for recruitment of patients is detailed in **Figure 4-1**.



Figure 4-1 Initial proposed pathway for patient recruitment in TRAFIC Study

4.5.5 After study approval

The final approval came in May 2019 and the Site Initiation Visit (SIV) took place in June 2019.

After approval and SIV, recruitment started under the study in July 2019. Our team commenced recruitment at a rapid pace with a success rate of about 75%. We made minor adjustments over the next few months to boost recruitment, based on our experiences on the ground. After a few months into recruitment, we held another Patient and Public Engagement symposium in October 2019 to take feedback from patients and make improvements. After implementing the feedback from patients, a final recruitment pathway was then generated and followed. This is illustrated in **Figures 4-2 and 4-3**

4.5.6 Recruitment

The recruitment of different cohorts of patients was driven by me and supported by the larger team of nurses and research coordinators. I undertook screening on a weekly basis for patients suitable to be approached for research. The nurses discussed the study with patients and recruited them if they consented. Blood samples and biopsies were taken as appropriate.

For the surgical patients, I was regularly in touch with the Colorectal surgeons and screened for patients at the weekly IBD Multidisciplinary Team (IBD MDT) meeting. When suitable patients were identified, I approached them with permission from the surgical team. I recruited patients who consented to be part of the study.

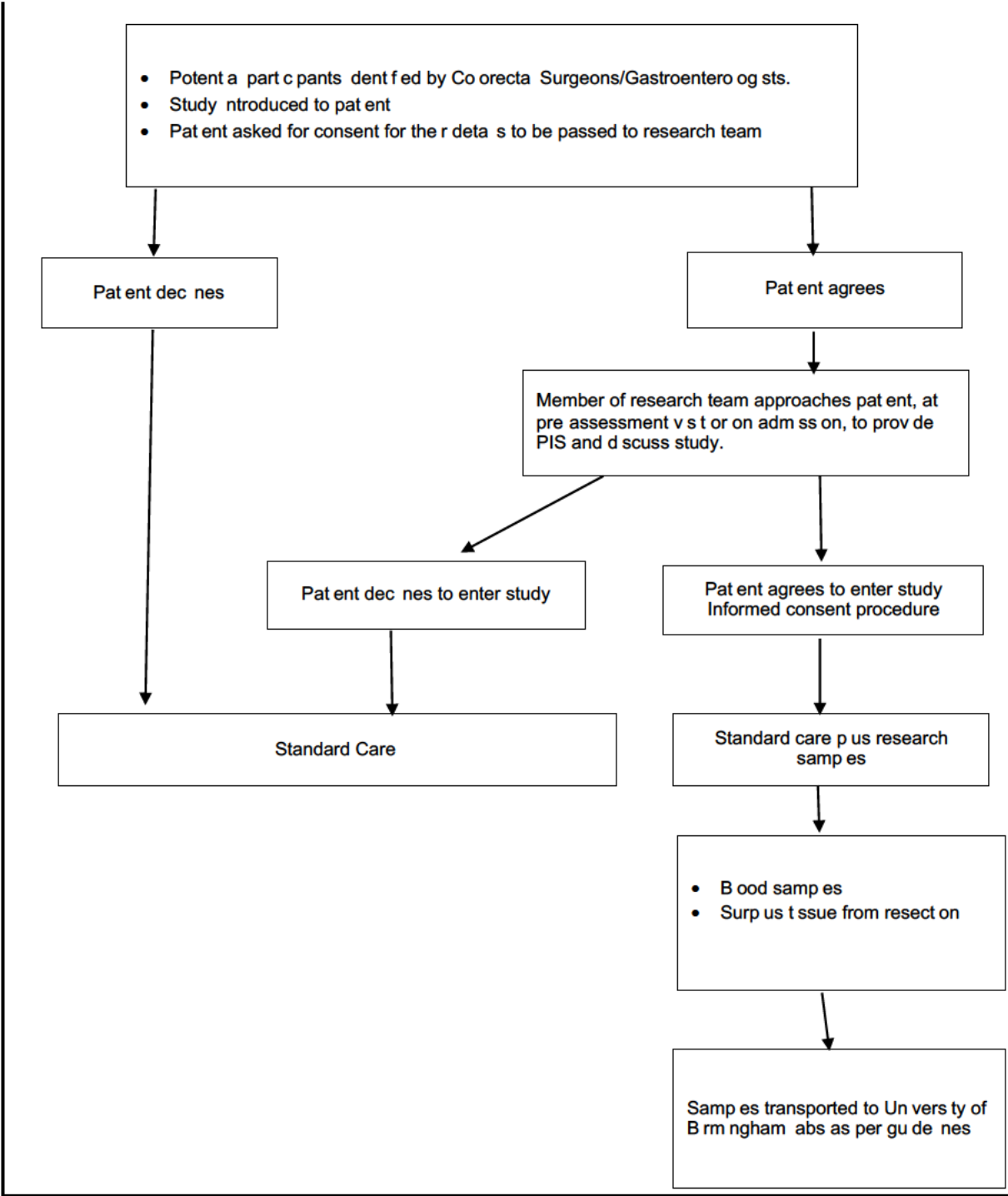


Figure 4-2 Final implemented pathway for patient recruitment (Surgical cohort)

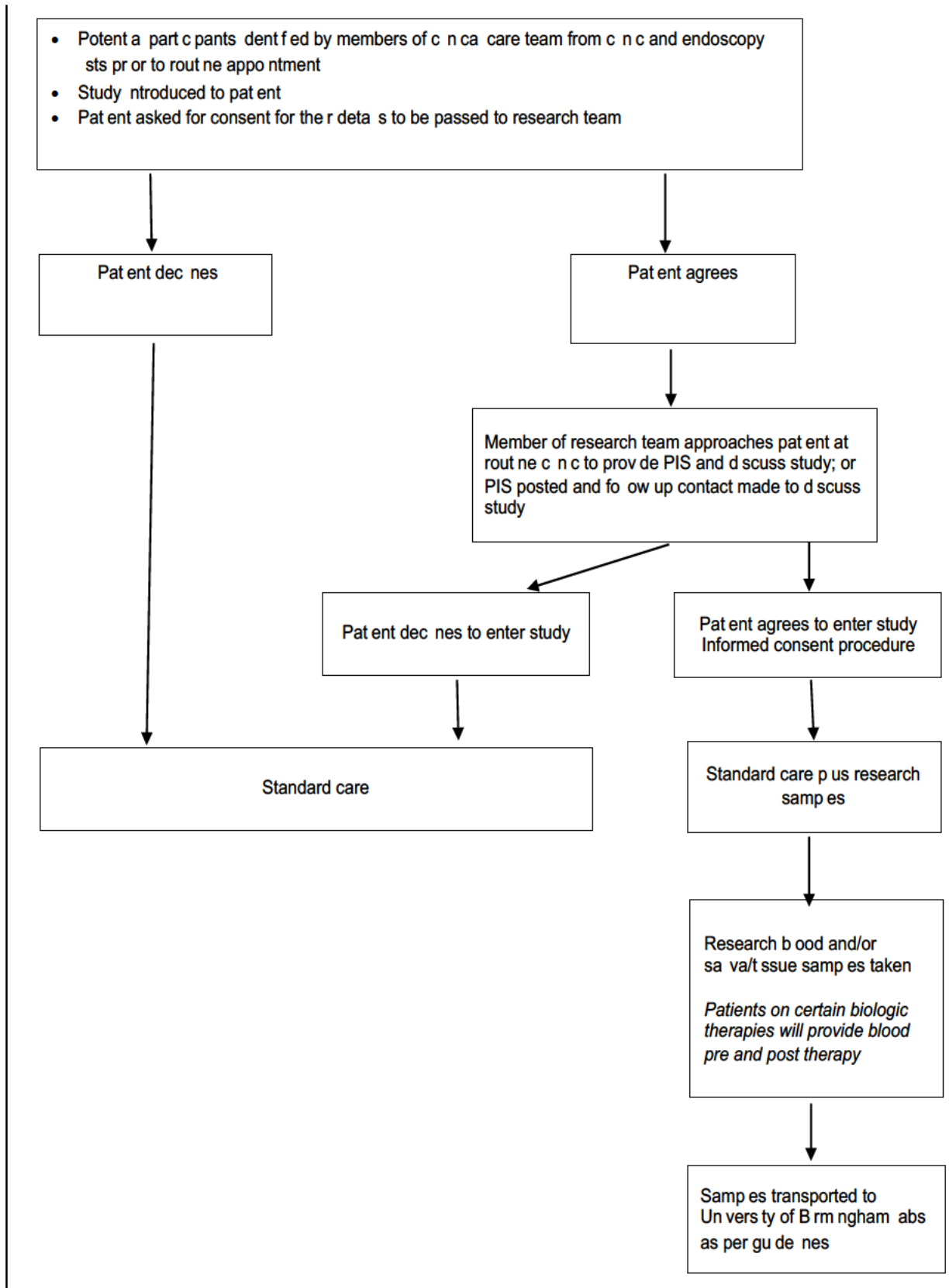


Figure 4-3 Final implemented pathway for patient recruitment (blood and biopsies)

4.6 Patients and Methods

One of the main stems of the study was the surgical cohort. 25 patients were initially recruited under this cohort. Of the 25 patients, 19 patients underwent surgery as planned from whom we were able to collect surgical resection tissue from all 3 segments of the resection sample.

The methods are described separately under -

- Methods on clinical side of study (recruitment and sample collection)
- Methods in the laboratory (sample handling, storage and processing)

4.6.1 Sample collection and transfer

The surgical tissue collection required coordination among multiple teams. As the interface between the surgeons, pathologists and laboratory team, I coordinated the communication about the dates and times of surgery. On the day of the surgery, it was critical that the samples obtained remained fresh to avoid hypoxic damage. To maintain the integrity of the tissue, the surgical team alerted me when the sample was ready to collect. I had access to the operating theatres where I would personally collect the tissue in a vacuum sealed pack and transfer it to the pathology department after various checks. I would then alert the pathologist about the transfer of tissue, so they were prepared to process it as soon as I arrived in the department.

Sampling in the pathology department

On my arrival in the pathology department with the resected sample, routine checks and registration process were undertaken. The pathologist and I would then examine the macroscopic specimen for margins, strictures and fistulae. The pathologist examined the tissue to ensure it was suitable to be sampled for the study. Once confirmed, sampling was done. From each patient, the aim was to collect tissue segments that were distal and proximal to the stricture in addition to the stricture itself as illustrated in **Figure 4-4**. The volume of tissue set aside for research was at the discretion of the pathologist. It was variable with each patient, usually commensurate to the overall size of the sample. The types of samples and pathology noted at dissection of macroscopic sample is illustrated in **Figures 4-5 to 4-9**. The size of stricture itself and any presence of other concerning features also influenced this decision. The size of tissue sampled ranged between 0.668-3.5 grams.

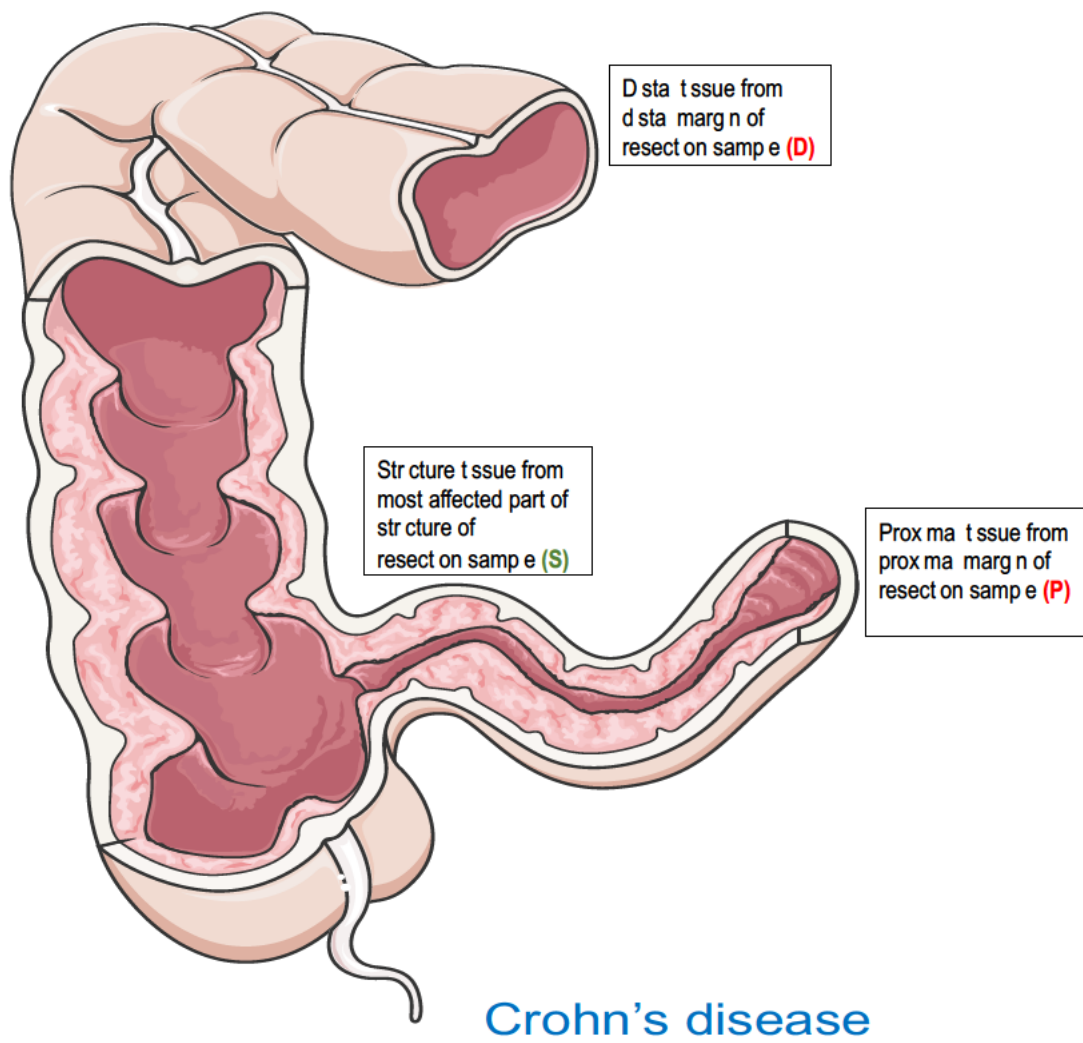


Figure 4-4 Illustration of sampling from the resected tissue of patient

(Figure adapted from Smart Servier)

Ileocolic resection from a patient with Crohn's disease showing fat wrapping



Figure 4-5 Macroscopic view of ileocolonic resection



Figure 4-6 Specimen of ileal stricture (blue arrow)

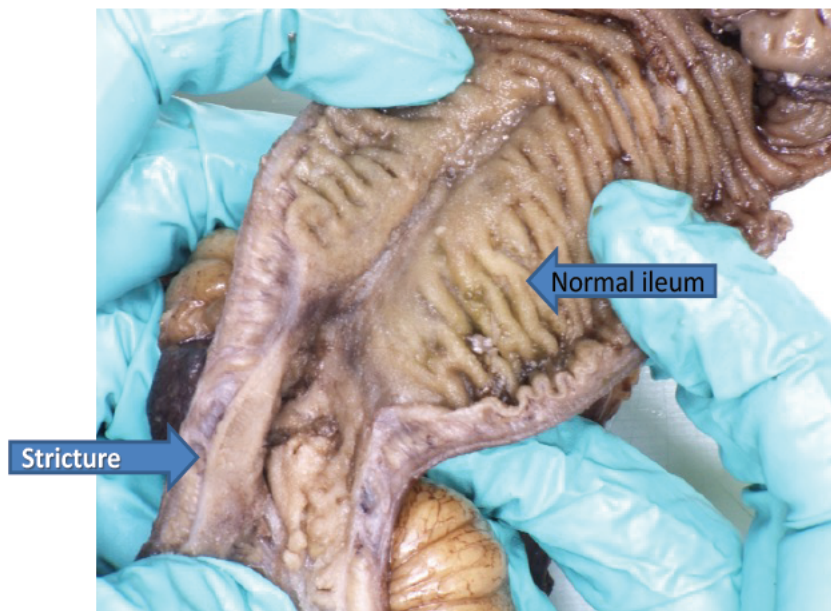


Figure 4-7 Specimen of stricture and normal ileum



Thickening of muscularis mucosa and extensive submucosal expansion

Figure 4-8 Demonstration of thickened muscularis layer and submucosal expansion in stricture

Tissue sample transport to labs

The tissue from each segment was collected and placed in separate containers labelled with the study code and origin of tissue e.g., (P)proximal, (D)distal and (S)stricture. The samples were placed into MACS tissue storage solution (Miltenyi Biotec, Germany), and transported immediately to the lab for further processing.

Blood sample transport to labs

Whole blood, matched to resected tissue, was collected on day of recruitment or on the day on of the surgery. Blood was collected in vacutainer tubes containing EDTA anticoagulant and labelled appropriately. They were transported securely as per our standard operating procedures.

We initially recruited 25 patients in the resection cohort. Of the 25, tissue from 19 patients was included in the final experiments recruited until the end of February 2020. 6 patients had to be excluded for various reasons (e.g., withdrawal of consent for surgery after recruitment in study, non-viable tissue for study due to formalin use in error, change in surgery on the operating table, and so on) After March 2020, recruitment was closed due to the pandemic. The demographics of and disease classification of all 19 patients is given in **Table 4-1**

Table 4-1 Demographics and disease classification of patients included

Lab code	Age	Sex	Years of diagnosis	Montreal Classification for age -CD	Montreal Classification for location-CD	Montreal Classification for behaviour
BRC-FCD-001	57	Male	8	A3 above 40 y	L3 ileocolonic	B3 penetrating p perianal disease modifier
BRC-FCD-003	54	Male	21	A2 between 17 and 40 y	L1 ileal	B2 stricturing
BRC-FCD-004	63	Female	38	A2 between 17 and 40 y	L3 ileocolonic	B2 stricturing
BRC-FCD-005	24	Male	16	A1 below 16 y	L1 ileal	B2 stricturing p perianal disease modifier
BRC-FCD-006	28	Male	6	A2 between 17 and 40 y	L1 ileal	B1 non-stricturing, non-penetrating
BRC-FCD-008	58	Female	24	A2 between 17 and 40 y	L1 ileal	B2 stricturing
BRC-FCD-009	19	Male	4m	A2 between 17 and 40 y	L1 ileal	B3 penetrating
BRC-FCD-010	35	Female	6	A2 between 17 and 40 y	L3 ileocolonic	B3 penetrating
BRC-FCD-012	55	Female	36	A2 between 17 and 40 y	L1 ileal	B2 stricturing
BRC-FCD-015	28	Female	19	A1 below 16 y	L1 ileal	B2 stricturing
BRC-FCD-016	37	Male	1	A2 between 17 and 40 y	L3 ileocolonic	B3 penetrating
BRC-FCD-017	39	Male	14	A2 between 17 and 40 y	L1 ileal	B2 stricturing
BRC-FCD-019	26	Male	12	A1 below 16 y	L3 ileocolonic	B3 penetrating
BRC-FCD-020	24	Male	10	A1 below 16 y	L3 ileocolonic	B3 penetrating p perianal disease modifier

BRC-FCD-021	55	Female	17	A2 between 17 and 40 y	L1 ileal	B3 penetrating
BRC-FCD-022	42	Female	7	A2 between 17 and 40 y	L3 ileocolonic	B2 stricturing
BRC-FCD-023	25	Female	6	A2 between 17 and 40 y	L1 ileal	B3 penetrating
BRC-FCD-024	48	Female	8	A2 between 17 and 40 y	L3 ileocolonic	B2 stricturing
BRC-FCD-025	56	Female	14	A3 above 40 y	L1 ileal	B2 stricturing

Sample handling for handover in the research laboratory

Resection tissue and blood samples were transported to the lab by a member of the lab team on duty for the day. Once the samples reached the lab, they were pseudo-anonymised on arrival by assigning a unique access number, as per existing lab protocols. A master log was maintained in the lab which contained both the study code and lab number (in case they needed reconciliation). The lab team were blind to the demographics and other clinical details.

Resection samples were weighed and depending on how much tissue was available, were further cut for various procedures. Tissue was placed in RNAlater for RNA extractions and part of it placed in formalin to generate FFPE sections. Tissue was also used for isolation of fibroblasts. The number of experiments planned and carried out varied, depending on the amount of tissue supplied.

Blood sample was processed to collect peripheral blood mononuclear cells (PBMCs) immediately for use in further experiments.

4.6.2 Methods in the laboratory

These are steps followed once the sample was handed over to the laboratory. All experiments were conducted in the Centre for Liver and Gastroenterology Research (CLGR) located in the Institute of biomedical research building at University of Birmingham.

For RNA analysis, 9 patients out of the 19 were selected. The demographics and disease classification of the final 9 patients included for RNA sequencing is given in **Table 4-2**.

Table 4-2 Demographics and disease classification of patients included for RNA seq

Lab code	Age	Sex	Montreal Classification for age -CD	Montreal Classification for location-CD	Montreal Classification for behaviour
BRC-FCD-003	54	Male	A2 between 17 and 40 y	L1 ileal	B2 stricturing
BRC-FCD-004	63	Female	A2 between 17 and 40 y	L3 ileocolonic	B2 stricturing
BRC-FCD-005	24	Male	A1 below 16 y	L1 ileal	B2 stricturing p perianal disease modifier
BRC-FCD-009	19	Male	A2 between 17 and 40 y	L1 ileal	B3 penetrating
BRC-FCD-010	35	Female	A2 between 17 and 40 y	L3 ileocolonic	B3 penetrating
BRC-FCD-012	55	Female	A2 between 17 and 40 y	L1 ileal	B2 stricturing
BRC-FCD-016	37	Male	A2 between 17 and 40 y	L3 ileocolonic	B3 penetrating
BRC-FCD-017	39	Male	A2 between 17 and 40 y	L1 ileal	B2 stricturing
BRC-FCD-019	26	Male	A1 below 16 y	L3 ileocolonic	B3 penetrating

Procedure for RNA extraction and preparation for sequencing

This process was collaborative along with the lab team. I was involved in processing the tissue for the first few patients. I was trained by my lab colleague and supervisor Dr. Louisa Jeffery on the steps involved in RNA analysis. I was able to undertake steps necessary for RNA extraction and observed the process of c-DNA library preparation. The later samples were handled and processed by Dr. Sarah Akiror with supervision from Dr. Louisa Jeffery and Dr. Evaggelia Liaskou. In total we processed tissue from 9 patients (**Table 4-2**) who had reliable volume of tissue available from all 3 segments (9x3=27), for RNA analysis. Each site was handled separately throughout, with appropriate labelling. For example, tissue from first patient was labelled BRC-FCD-001(S) to denote stricture site, FCD-001(P) to denote proximal margin and FCD-001(D) to denote distal margin, and so on for all patients.

The various steps involved in RNA analysis are described in this section.

RNA extraction from cells

All reagents were provided in the kit except for ethanol solutions prepared in the lab. Total RNA was extracted from lysed cells using the RNEasy micro kit (Qiagen, UK) according to manufacturer's instructions. The RNEasy micro kit is designed to extract RNA of up to 45 µg or in samples with cells <500,000, therefore the kit was fit for purpose based on the seeding density of cells used in the in vitro assays. Lysed cells were homogenised and 70% of ethanol (Fisher Scientific, UK) was added to precipitate the RNA from the cell lysates which was then collected

within the membrane of the RNeasy MinElute spin column through centrifugation. Wash steps were performed followed by elimination of genomic gDNA using Deoxyribonuclease I (DNase I). Further wash steps were performed to purify RNA, with RW1, RPE buffers and 80% ethanol (Fisher Scientific, UK) used for these wash steps. These solutions were used to eliminate carbohydrates, proteins, fatty acids and residual salts retained in the column. The membrane was dried by centrifugation at maximum speed (12000 x g for 5 minutes). RNA was then eluted in 14 µL RNase-free water by centrifugation at maximum speed for 1 minute. The concentration of eluted RNA in ng/µL was measured using a Nanodrop Spectrophotometer (ThermoFisher Scientific, UK). The absorbance ratios at 260 nm/280 nm and 230 nm/260 nm were also recorded to check the purity of RNA and contaminants that may have resulted from reagents used. For an RNA sample a 260/280 ratio of >1.8 is considered pure RNA and a 260/230 ratio of >1.5 is considered clean of organic solvents.

Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was synthesised using iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) according to manufacturer's instructions. Extracted RNA (up to 1 µg) was used as the starting RNA template. This was transcribed into cDNA by a mixture of iScript reverse transcriptase, iScript reaction mix which contained oligo(dT) and random hexamer primers and nuclease free water to a total volume of 20 µL, as detailed in the

Bulk RNA sequencingRNA extraction

Total RNA was extracted from 9 matched gut resected tissue samples (proximal, stricture and distal segments) that had been stored in RNAlater (Sigma-Aldrich, Gillingham, UK) using the RNEasy mini kit (Qiagen, UK) according to manufacturer's instructions. The RNEasy mini kit is designed to extract RNA of up to 100 µg from cells or tissue. Tissue was transferred from RNAlater solution to a gentleMACS™ C tube containing RLT lysis buffer containing β- mercaptoethanol, and then the tissue was disrupted using a pre-set protocol "RNA_01 for tissue" on the gentleMACS™ dissociator. Further tissue disruption was performed using a 10 mL syringe, mixing tissue "up and down" to obtain a homogenous solution. Disrupted tissue was then centrifuged for 5 mins at 12000 x g for 5 minutes, after which the supernatant was transferred to a fresh tube while the pellet was discarded, and 70% of ethanol (Fisher Scientific, UK) was added to the supernatant to precipitate the RNA.

The homogenised sample including any precipitate was transferred to an RNeasy Mini spin column, and centrifugation was performed at ≥8000 x g. A wash step was then performed using the RW1 buffer followed by elimination of genomic gDNA using Deoxyribonuclease I (DNase I). Further wash steps were performed to purify RNA, with RW1 and RPE buffers (Qiagen, UK) were used for the wash steps. These solutions eliminate carbohydrates, proteins, fatty acids and residual salts stuck in the column. The membrane was dried by centrifugation at maximum speed (12000 x g for 5 minutes). RNA was then eluted in 30 µl RNase-free water by centrifugation at maximum speed for 1 minute ready for the next experimental steps.

RNA quality assessment

Extracted RNA was subjected to quality checks: RNA concentration and purity (A260/A280) was measured using the Nanodrop Spectrophotometer (ThermoFisher Scientific, UK) while RNA quality and integrity was checked using the 2100 Bioanalyzer (Agilent) by measurement of the RNA integrity number (RIN). Samples with purity of >1.8 at OD260/280 and RNA integrity number (RIN) of >7 were used to prepare cDNA libraries.

cDNA library preparation for sequencing

Preparation of cDNA libraries was performed using the QIAseq UPX 3' (Qiagen, UK) according to manufacturer's instructions. In summary, 0.5ng of RNA was reverse transcribed to cDNA; during this step each sample was tagged with a unique molecular index (UMI). All samples with tagged UMIs were then pooled into a single tube and subjected to further amplification through PCR using the Qiagen HiFi PCR master mix (Qiagen, UK). The amplified cDNA sequence was fragmented into uniform short reads to make sequencing possible; the fragmented ends were repaired and "A-tailed" by addition of an adenine base. The library was completed by ligation of adaptors to the cDNA fragments, that serve to identify the library and attach it to the flow cell during the sequencing process. Finally, the cDNA fragments each tagged with their UMI and library adaptors were further amplified by PCR to enrich the amplicon and increase sensitivity of the assay. The amplified fragments in the cDNA library were quantified using the QIAseq Library Quant Assay Kit (Qiagen, UK) and sequenced using the Nextseq Illumina platform. Nextseq Illumina sequencing was performed by the genomics department within the Institute of Biomedical Research, University of Birmingham. Raw data was generated as FastQ files ready for analysis.

4.6.3 Methods and Results of bulk-RNA sequencing data (Bioinformatics)

Broad description of analysis

This was collaborative work that I did along with the lab team and bioinformatics team. Bulk RNA-seq data analysis was performed by Dr Animesh Acharjee, a senior bioinformatician in Institute of Cancer and Genomic Sciences, University of Birmingham. Dr. Acharjee has extensive experience with a sub-specialty interest in 'omics' data analysis. We had several rounds of discussion during the analysis stage to discuss the relevance of results. The FastQ files obtained as raw data were de-multiplexed, aligned to the genome, quantified and normalised using the CLC Genomics workbench (Qiagen, UK). The normalised values of reads reported in transcripts per million (TPM) were quality controlled by excluding genes with transcript expression of mean normalised counts that were less than a set threshold (< 1) and a false discovery rate corrected p value of < 0.05 was considered and filtered for further downstream data analysis. Partial least squares discriminant analysis (PLS-DA) modelling was performed on these genes and a Variable Importance in Projection (VIP) score was used as a filter of genes of importance as per the model, genes with VIP Score of ≥ 1 were considered to be important in the PLS-DA model. Differentially Expressed Genes (DEGs) were identified using the Linear models for Microarray data analysis (limma) package. Gene expression was compared between proximal vs stricture (P vs S), distal vs stricture (D vs S) and proximal vs distal (P vs D). Pathway enrichment was done using the databases Gene ontology biological pathways, Wiki pathways and Kyoto Encyclopaedia of Genes and Genomes (KEGG).

Detailed description of methodology of analysis*Step 1*

The FastQ sequencing files were obtained through BaseSpace (Illumina), and reads were de-multiplexed, cleaned, aligned, quantified, and normalised to human genome using the CLC Genomics Workbench (Qiagen). Quality control (QC) was performed on the data by filtering out lowly expressed genes (≤ 1 transcript), and all samples passed the QC process; filtered data was then subjected to linear models for microarray data (limma). Limma is a bioconductor package written in R and used for identification of differentially expressed genes (DEGs) to investigate transcriptional changes and differences in gene expression profiles in the stricture (S), proximal (P) and distal segments (D). Pathway enrichment was done using the databases Gene ontology biological pathways, Wiki pathways and Kyoto Encyclopaedia of Genes and Genomes (KEGG).

Step 2

To study variation in gene expression of the 3 segments, a total of 501 DEGs were analysed by principal component analysis (PCA). PCA is a univariate regression analysis model that is used to show trends and similarities in the dataset based on gene expression patterns; clusters are formed based on similarities in gene expression profiles. PCA reduces dimensionality of the data into principal components and is visualised as a 2-dimensional (2D) graph displayed as principal component 1 (PC1) vs principal component 2 (PC2); PCA is used to cluster samples into different groups based on their location in the PC1 vs PC2 plot, each PC is independent of the other. Principal components are linear combinations of the data's original variables; PC1 is the matrix projection containing features with the largest variance along the first axis. For examples, PC1

contains a combination of genes that contribute to the highest variation within samples along the PC1 axis. Principal component 2 (PC2) contains a combination of genes that result in the second largest sample variation within the data set. Therefore, PC1 vs PC2 plots show the highest spread of the data resulting in better recognition of similarity or dissimilarity in gene expression profiles across samples (457).

Figure 4-9A shows unsupervised PCA plots in which the training data set for the analysis algorithm was not provided with specific information (labels) or variables about the data. The PCA analysis scores show a 47.2% (x-axis) and 10.3% (y-axis) variance as per PC1 vs PC2 respectively. Samples from the stricture clustered together and clearly separated from the non-stricture (proximal+distal). The non-stricture segments also formed a discrete cluster suggesting a similarity in gene expression profiles.

Step 3

In the next step, supervised PCA analysis was performed where labels and specific information about the data set such as the group to which the samples belong (stricture, proximal and distal) is input during training of the algorithm. As illustrated in **Figure 4-9B**, the PCA plots showed a 47.1% (x-axis) and 5.8% (y-axis) variance for PC1 and PC2 respectively. Supervised PCA plot showed tighter clustering from the proximal and distal segments as compared to stricture. A wide range of variability in gene expression within the stricture samples was also observed. It is possible this could reflect different stages of fibrosis and different pathways being active.

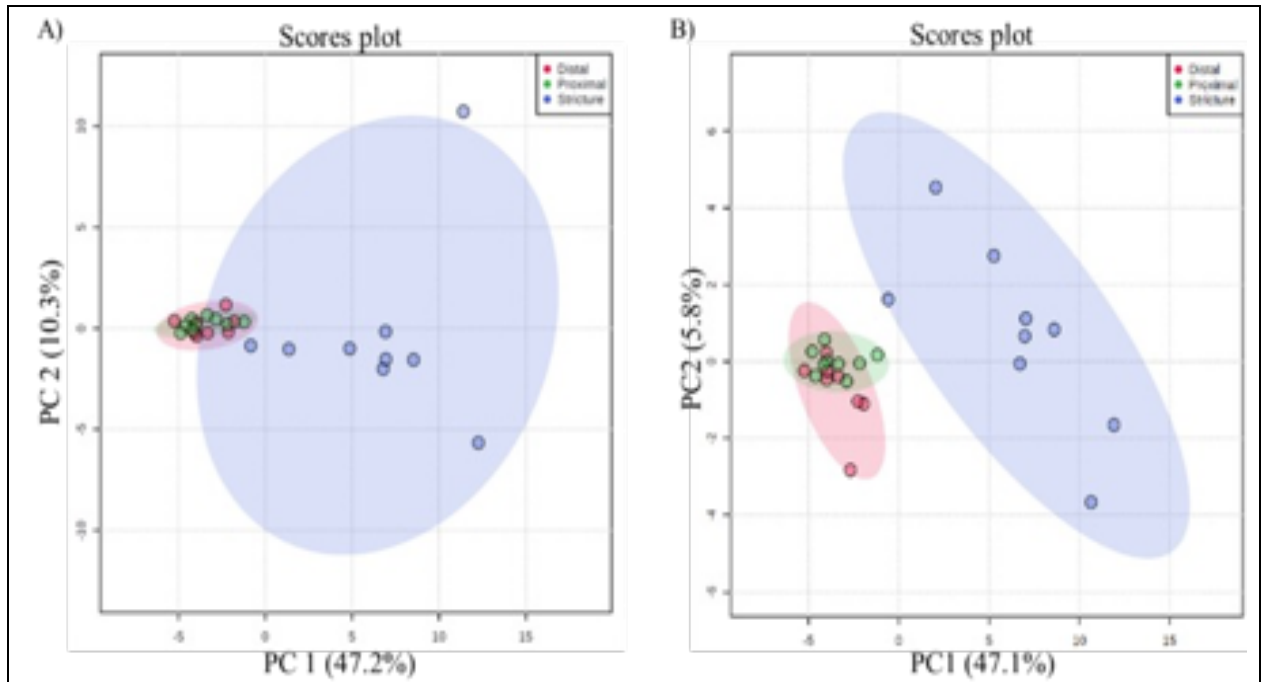


Figure 4-9 Principal Component Analysis (PCA) of differentially expressed genes

Transcriptome analysis showed differential gene expression profiles in the stricture, proximal and distal segments of resected gut.

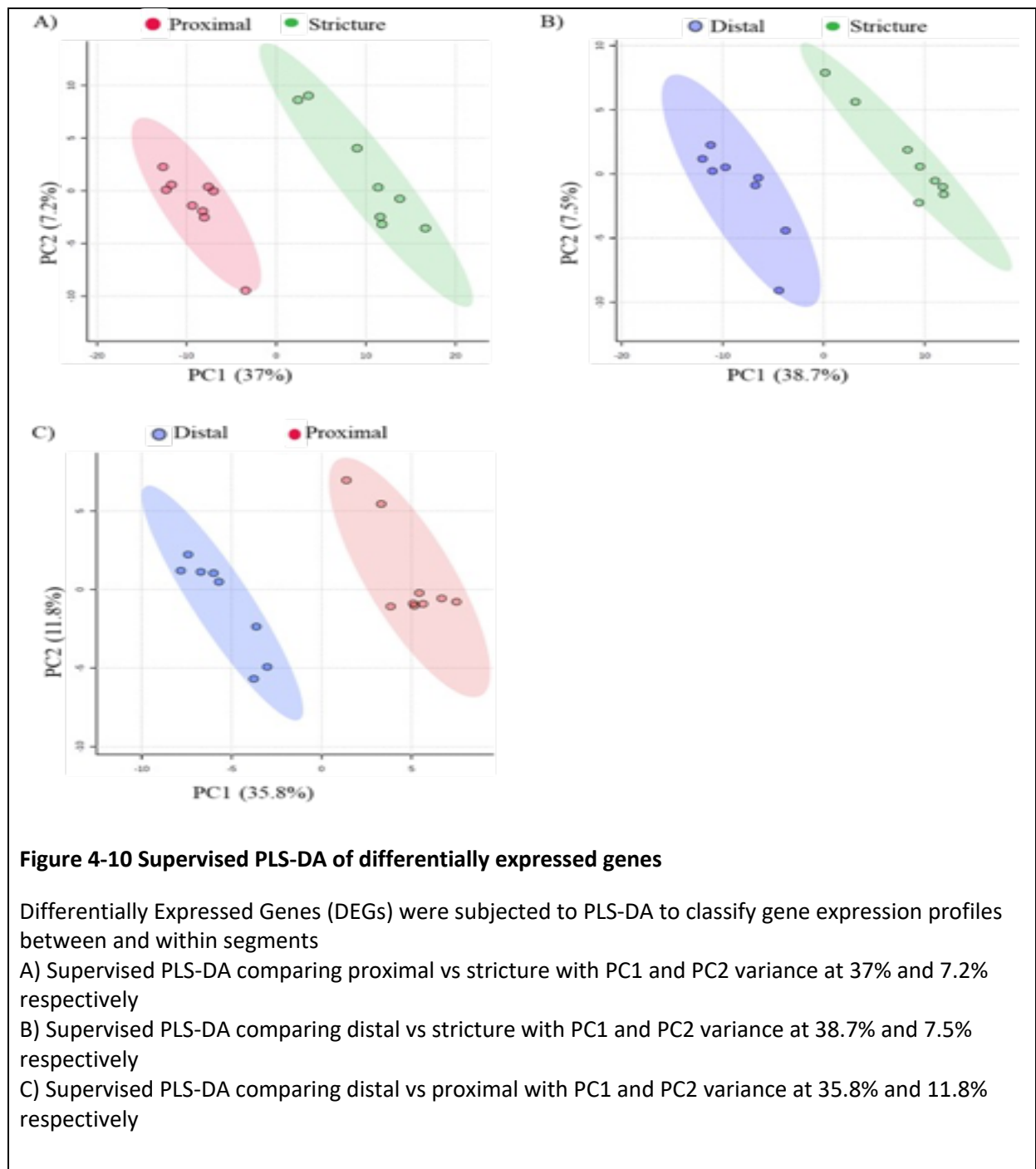
A) Unsupervised principal component analysis (PCA) showing an overview of gene expression patterns in the proximal (green circles), stricture (blue circles) and distal segments (red circles).

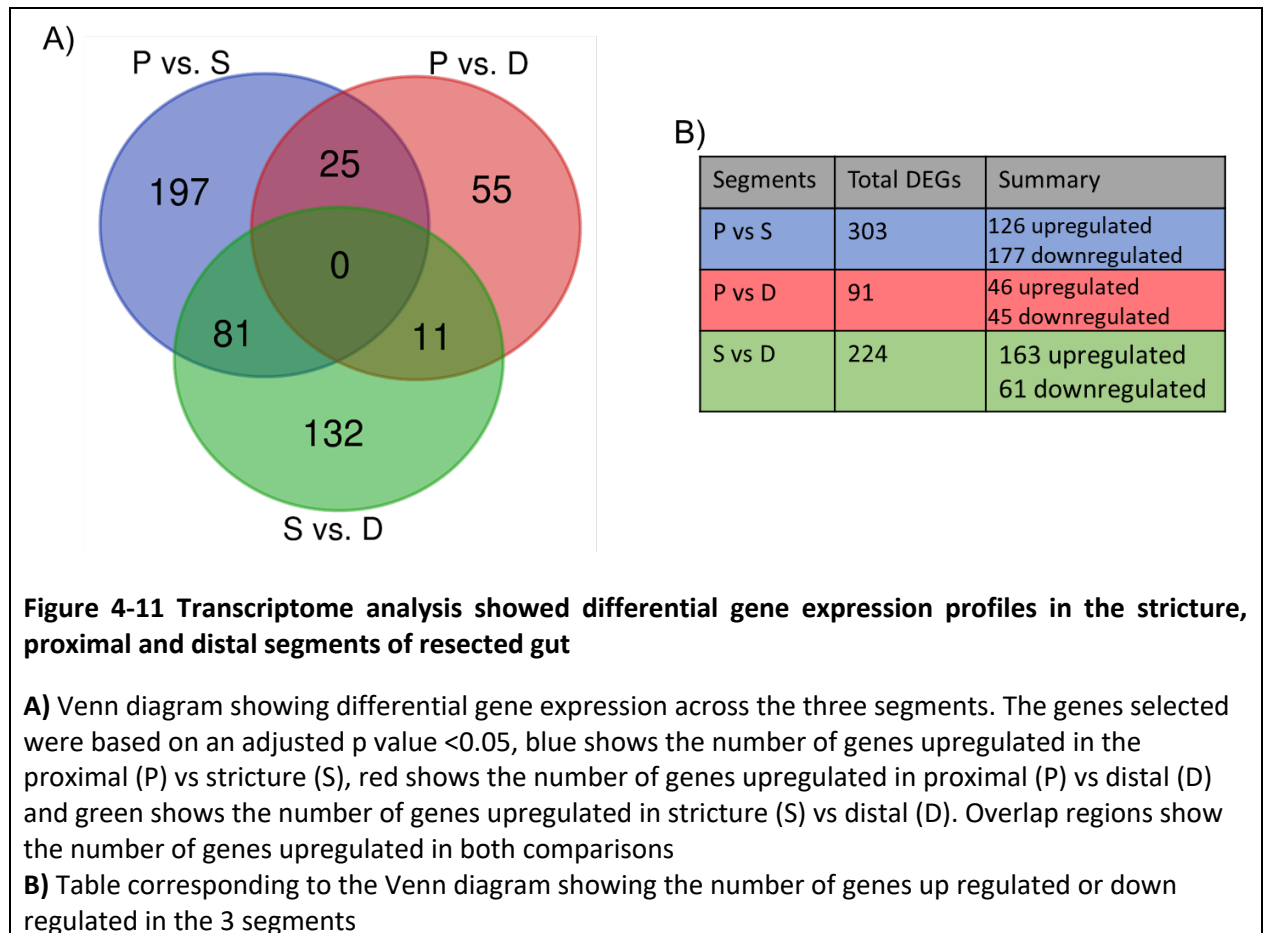
B) Supervised PCA showing differences in gene expression of the stricture (blue circles) to proximal (green circles) and distal (red circles). Gene expression of non-strictured segments appear similar in their pattern of gene expression and vary from the strictured segment.

Step 4

To study gene expression patterns in samples between and within each segment, pairwise supervised partial least-square discriminant analysis (PLS-DA) was performed. PLS-DA is a multivariate regression analysis model that is applied for discrimination of multiple variables according to their gene expression profiles. PLS-DA plots and the variance are illustrated in **Figures 4-10 A, B and C**. The analysis shows distinct gene expression patterns between each segment of the bowel.

With the application of these methods, a total of 501 differentially expressed genes (DEGs) were identified in the 3 segments. A detailed breakdown of this is illustrated in **Figure 4-11**.





Step 5

With the number of genes now narrowed down further, the next step was to explore the transcriptome profiles within the stricture. DEGs were identified that were regulated in both the proximal versus stricture and stricture versus distal comparisons. They were selected and subjected to hierarchical clustering. This is visualised as a heat map as seen in **Figure 4-12**.

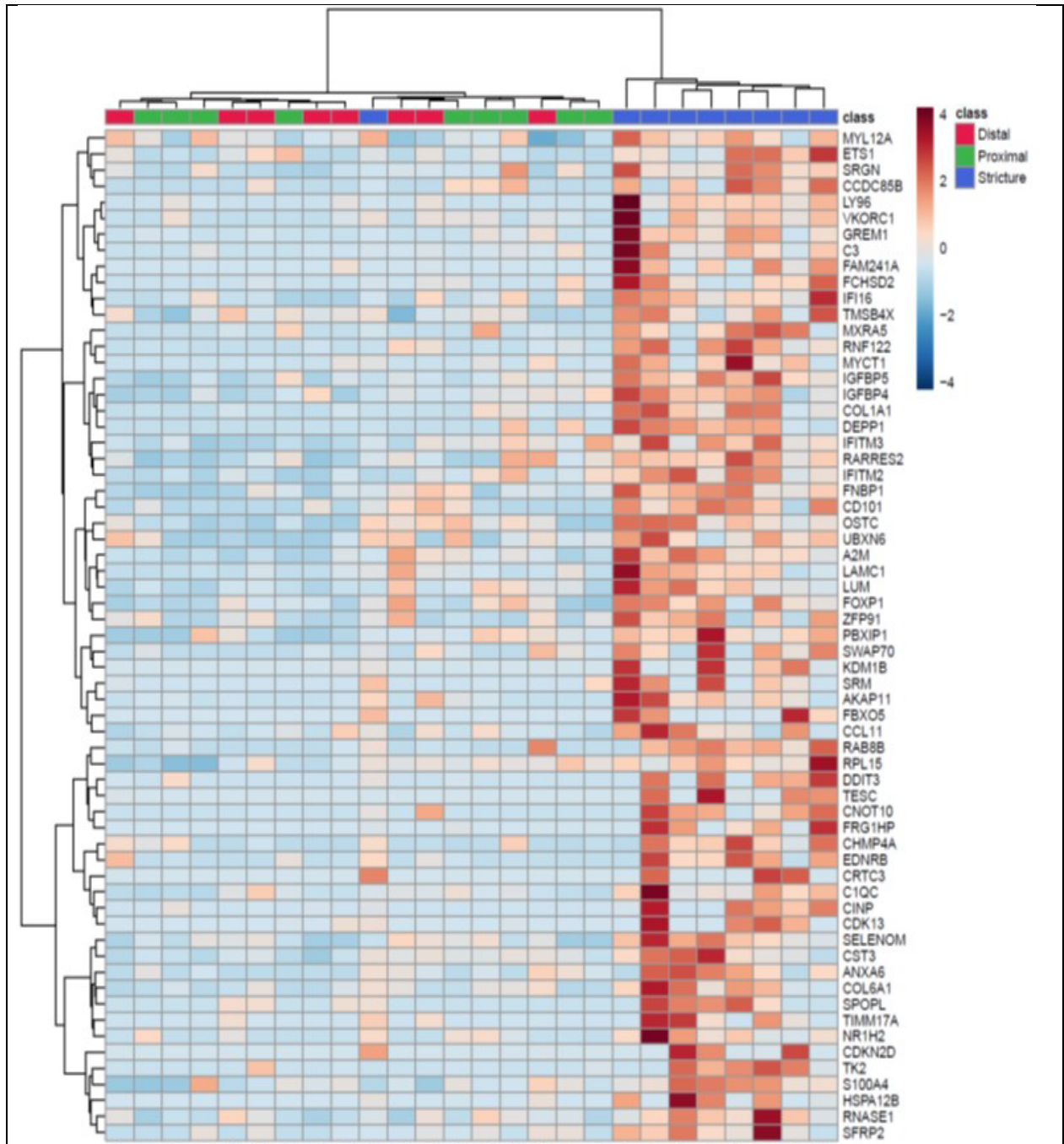


Figure 4-12 Hierarchical clustering of differentially expressed genes to classify gene expression patterns in the stricture and non-stricture segments

The DEGs shown in the heat map were selected from the pairwise comparisons of segments (P vs S and S vs D). Gene expression profiles were analysed by hierarchical clustering in samples from the proximal (green), distal (red) and stricture (dark blue). The colour range indicates expression level - from deep red (high levels in gene expression) to blue (low levels in gene expression)

Hierarchical clustering revealed a separation in gene expression profiles between stricture and non-stricture segments. There was a unique transcriptomic fingerprint of fibrotic related genes whose expression was upregulated in samples from the stricture segment. This suggests a distinct fibrotic molecular signature within the stricture segment. The heat map in **Figure 4-12** also showed a clustering pattern in which samples from each segment (stricture and proximal) cluster together.

Step 6

Once the DEGs were narrowed down, we had a discussion to understand how to interrogate the most relevant genes. We first ranked genes in the final list of DEGs with a cut off set at a p-value <0.05 and then examined the biological association of each with respect to pathways that drive a profibrotic phenotype. After reaching a consensus, a group of genes *LY96*, *GREM1*, *SERPINE1*, *HDAC1* and *FGF2* were selected as predictors of stricturing disease. To test model performance for prediction of stricturing, AUC (Area Under the Curve) and confidence interval were calculated. AUC values measure overall accuracy of a model for prediction based on sensitivity and specificity where the higher the sensitivity and specificity, the higher the accuracy. (a value closer to 1 is more accurate)

In this analysis *GREM1*, *SERPINE1* and *FGF2* showed the highest potential to predict intestinal tissue stricturing with AUC of *GREM1* (0.88), *SERPINE1* (0.77), *FGF2* (0.7). *HDAC1* and *LY96* had lower AUC values of 0.65 and 0.69 respectively. When combined, these genes gave a high predictive AUC value of 0.81 at the 95% confidence interval.

Further work on quantitative PCR analysis of the selected genes was carried out by the lab team, which formed bulk of the work for the thesis of one of my colleagues, Sarah Akiror (Dr). There are some overlaps in parts of this common analysis.

Following this comprehensive initial analysis, my focus was to examine some targets of specific interest, selected based on recent literature on fibrosis. After discussion with supervisors, I divided the work into two main parts-

- Interrogation of pre-selected pathway (JAK-STAT pathway)
- Immunohistochemistry (IHC) of selected cytokines (IL-33, IL- 36gamma and TNF alpha)

The next section includes the results and discussion of the JAK-STAT pathway followed by IHC.

4.7 JAK-STAT pathway related gene expression

Background of JAK-STAT pathway

The Janus Kinase (JAK) signal transducer and activator of transcription (JAK-STAT) pathway is an evolutionarily conserved signalling pathway that transmits extracellular signals to the nucleus to regulate gene expression. It has several crucial functions in physiological processes. (Xue, Yao et al. 2023) Structurally, the JAK-STAT pathway involves two main components. First are the transmembrane receptors, receptor-associated cytosolic tyrosine kinases (i.e., JAKs). Second are the downstream signal transducers and activators of transcription (i.e., STATs). The JAK family contains four members: JAK1, JAK2, JAK3, and Tyrosine Kinase 2 (TYK2). The STAT family consists of seven proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. (Figure 4-5)

The JAK-STAT pathway was discovered in the early 1990s when researchers were studying how IFNs led to activation of a transcription factor. Over the next few years several laboratories started reporting on different members of the JAK and STAT family. (Xue, Yao et al. 2023) The therapeutic potential of this pathway was later reported by multiple groups over the next 2 decades. (Darnell 2002, Chen, Daines et al. 2004)

The mechanisms of signalling via the JAK-STAT pathway have been well described. The JAK receptors at the cell membrane are activated by its ligand. Upon ligand binding, receptors dimerize and activate JAKs, which subsequently phosphorylate the STAT proteins, allowing them to dimerize, translocate to the nucleus, and modulate gene expression. This cascade is central to many biological processes. More than 50 types of cytokines, including interferons (IFNs), interleukins (ILs), and growth factors, have been shown to play roles in JAK-STAT signalling to fulfil regulatory functions in cell differentiation, metabolism, survival, homeostasis, and immune response. (Xue, Yao et al. 2023)

The components in this pathway perform multiple functions and they are regulated by other factors, which keep the balance of positive and negative regulation. When this balance is disrupted, they could potentially be involved in many disease conditions also. JAKs and STATs have been known to be part of pathogenesis of many conditions and have also been exploited as targets of therapy. The multiple functions, ligands, positive and negative regulators of JAKs are illustrated in **Figure 4-13**. The members of the STAT family too have unique functions and have been implicated in many conditions.

There are many regulators of the JAK-STAT pathway, most important of which is the Suppressor Of Cytokine Signalling (SOCS) family. With this regulation, the pathway performs multiple complex functions. When stimulated by different cytokines, the JAK-STAT signalling pathway plays a role in immune regulatory events, that include not only cancer cell recognition, triggered mainly by IFNs and STAT1 and STAT2 signalling, but also immune escape triggered mainly by IL-6-STAT3 signalling. (Xue, Yao et al. 2023) It has been widely shown to drive antitumor immune surveillance in response to cytokines (like IL-2, IL-15, and IFNs) by inducing the activation, cytotoxicity, and function of natural killer (NK) cells. (Kang, Biswas et al. 2019) On the other hand, the JAK-STAT pathway has been implicated in the pathogenesis of numerous autoimmune diseases such as, IBD, Rheumatoid Arthritis (RA), and Atopic Dermatitis. IL-6 induced the phosphorylation of STAT3 and upregulated the differentiation of Th17 cells is known to be contributory to RA. (Xue, Yao et al. 2023) Genome-wide association analyses have demonstrated that polymorphisms and mutations in the JAK-STAT pathway are associated with autoimmune diseases and immune-mediated cancers. (Ellinghaus, Ellinghaus et al. 2012) There are many reports of STAT3 and STAT6 mutations involved in autoimmune disorders. The identified role of STATs in many conditions are illustrated in **Figure 4-14**.

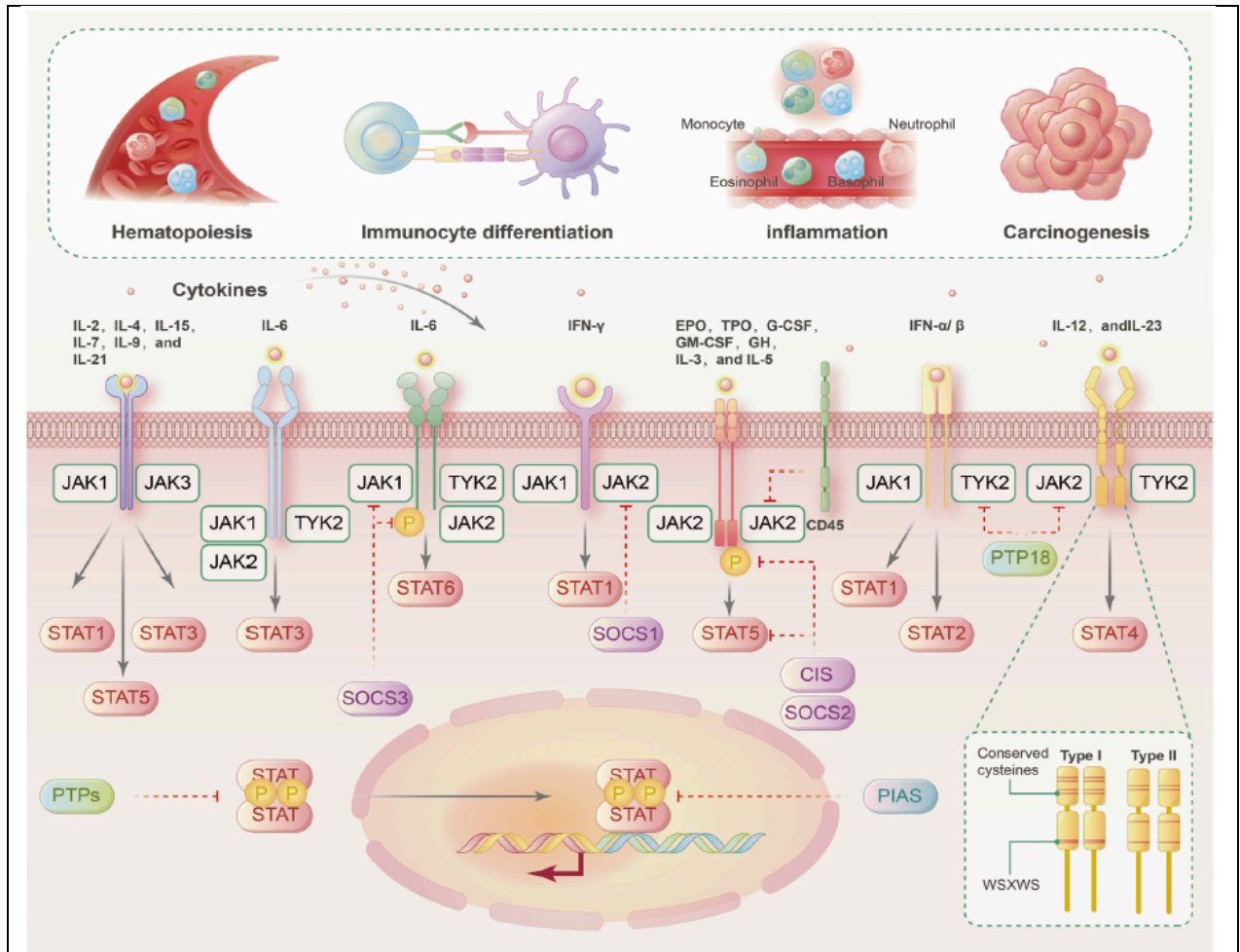


Figure 4-13 Canonical pathway of JAK-STAT signalling

Canonical activation and negative regulation of JAK-STAT signalling pathways. Canonical activation of the JAK-STAT signalling pathway: cytokines bind to their corresponding receptors, which undergo a conformational change and recruit related JAKs. The JAKs undergo phosphorylation, which leads to tyrosine phosphorylation of the receptors to create docking sites for STATs. STATs are phosphorylated, dissociate from the receptor, and enter the nucleus as homodimers or heterodimers to bind specific DNA sites and regulate cytokine-related gene transcription. Negative regulation of the JAK-STAT signalling pathway: the CIS/SOCS (cytokine signalling inhibitor), PIAS (protein inhibitor of activated STAT), and PTP (protein tyrosine phosphatase) families are participants in the negative regulation of the JAK-STAT signalling pathway. The CIS/SOCS family negatively regulates the JAK-STAT pathway through direct binding of JAKs or JAK receptors to inhibit JAK kinase activity, binding of receptor tyrosine kinase sites to intercept STAT-receptor binding, or formation of enzyme complexes to degrade JAKs or STATs. The PIAS family mainly interacts with STAT dimers to inhibit STAT binding to DNA, thereby blocking JAK-STAT signal transduction. The PTP family negatively regulates the JAK/STAT pathway mainly by dephosphorylating activated receptors, JAKs, and STATs. Solid lines indicate the activation process. The dotted lines represent negative regulation (Figure and legend used with permission from Xue et al open access article)

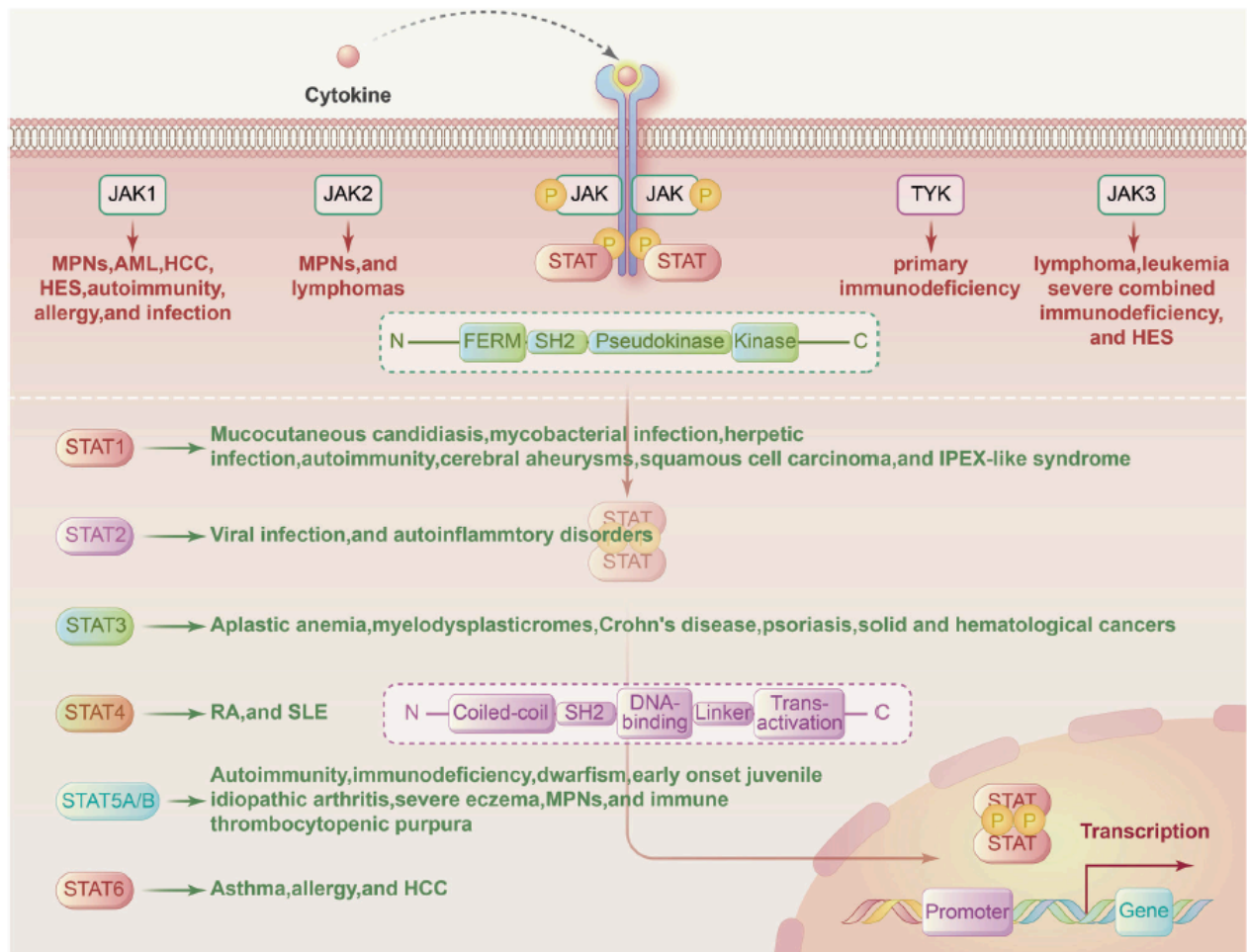


Figure 4-14 Types of autoimmune disorders involving JAKs and STATs (includes mutations and polymorphisms)

(Figure and legend used with permission from Xue et al open access article)

With this knowledge, the JAK-STAT pathway has been exploited as a target of therapy in recent years. Recent clinical trials have shown that targeting JAKs and preventing their phosphorylation suppresses abnormal immune and inflammatory responses caused by cytokines, providing solid evidence to support the use of JAK inhibitors as therapies to treat autoimmune diseases and cancers. (Xue, Yao et al. 2023) There are now multiple JAK inhibitors (JAKinibs) available for the treatment of autoimmune diseases and cancer.

Oral small molecule JAKinibs in IBD

In IBD, the prototype pan-JAKinib first approved for therapy was Tofacitinib. It was shown to be successful in clinical trials in managing UC (Sandborn, Su et al. 2017), but not effective in CD. Since then, other, more selective JAKinibs have been approved for therapy in UC such as filgotinib and upadacitinib. (Feagan, Danese et al. 2021, Danese, Vermeire et al. 2022) More recently upadacitinib has been reported to be effective in managing CD and has been approved for therapy. As they are oral small molecules, they have some advantages in treatment, but they do have some disadvantages too. These are listed in **Figure 4-15**.

	Potential advantages	Potential disadvantages
Route of administration	Improved patient compliance; cheaper to transport and store	Dependence on the patient for ensuring compliance; might add to pill burden in older patients with polypharmacy
Short half-life	Start-stop strategy; short washout periods	Daily or multiple daily dose regimen intake of drug
Immunogenicity	Useful in patients who are known to mount an immunogenic response without the need to use immunomodulators such as thiopurines	None
Off-target effects	None	Unanticipated side-effects that might be difficult to manage
Narrow dosing windows	Substantial dose adjustment unnecessary	Limited opportunity to dose escalate
Dosing and dose escalation	Easy to escalate within dosing window	Daily and multiple dosing; limited scope to escalate dose; uncertain bioavailability at target site and clearance affected by renal or hepatic impairment; drug-drug interactions

Figure 4-15 Advantages and disadvantages of oral JAK inhibitors

(Table adapted with permission from Shivaji et al)

In IBD, all JAKinibs exert their action by blocking cytokine driven signalling via the JAK-STAT pathway. There have been no reports specifically studying their effect on intestinal strictures in IBD. Although TYK inhibitors have shown anti-fibrotic effect in lung and liver fibrosis, it has not yet been extended to manage intestinal fibrosis. There are drugs already approved for therapy, such as nintedanib for Idiopathic Pulmonary Fibrosis (IPF) (Richeldi, du Bois et al. 2014, Fletcher,

Jones et al. 2018). There are multiple other drugs currently in clinical trials for IPF and hepatic fibrosis. (Shan, Wang et al. 2022) (Qu, Huang et al. 2016) JAKinibs have been tested for antifibrotic potential but so far none have shown any promise in intestinal fibrosis. However, they remain a popular group as their mechanism is likely to hold potential, and this group of drugs forms 25% of all antifibrotic therapies being investigated in clinical trials across all specialties. (Lin, Mao et al. 2022) These drugs which carry anti-fibrotic potential may have an important role to play along with other existing therapies for IBD. It is possible they may be used as adjunct therapies along with anti-inflammatory agents thereby maximising therapeutic effect and prevent formation of strictures. This potential effect needs to be investigated further.

Rationale for additional analysis of transcriptomics data

From the studies reported so far, it is evident that JAKinibs are useful targets of therapy to control inflammation in IBD, but it may hold potential benefits as an antifibrotic. We wanted to study the JAK-STAT pathway in our cohort of patients, given that they all had stricturing phenotype (B2) as the cause for surgery.

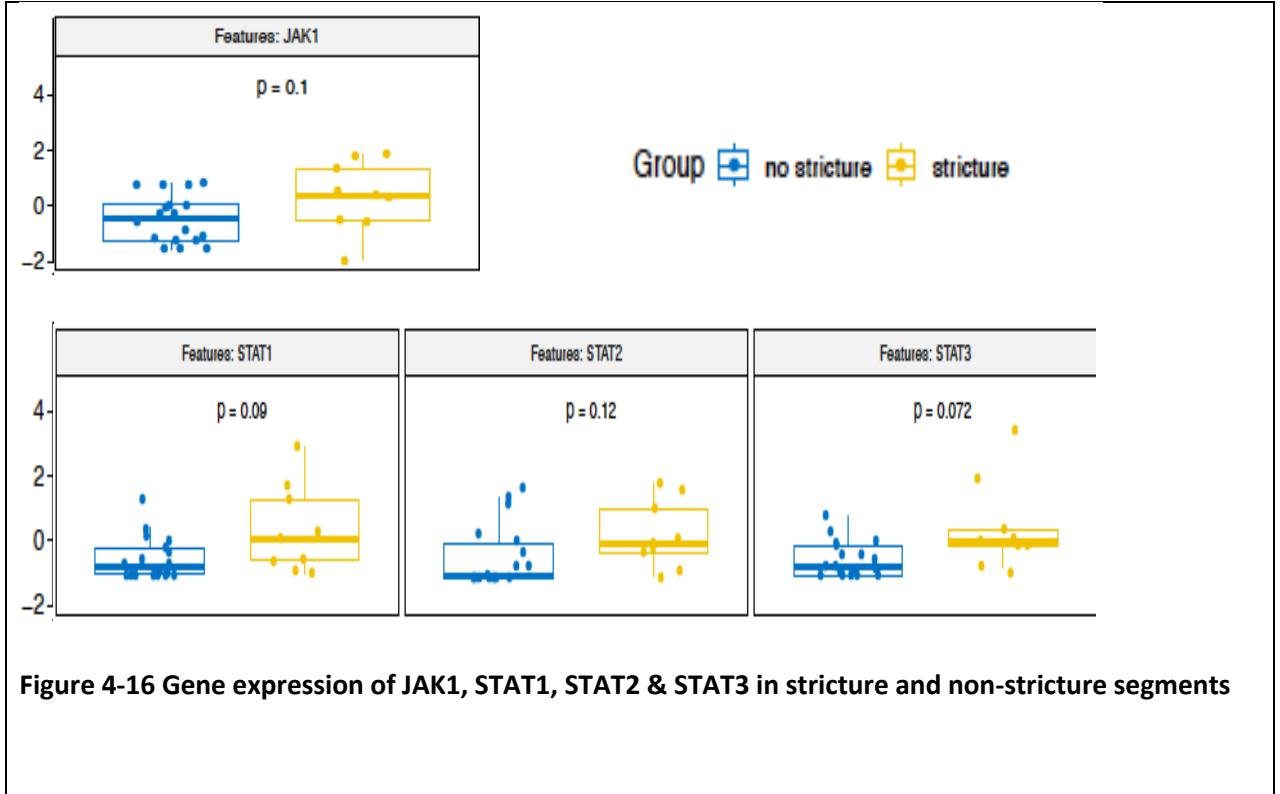
Methods of analysis

The bulk RNA sequencing data that was generated using methodology described in the previous sections was again used to conduct further detailed analysis by selecting specific components of the JAK-STAT pathway. Whereas the first round of analysis used methodology as described earlier, for the JAK-STAT pathway, we pre-selected the most relevant genes of interest within this pathway. If there was no gene expression at all (zero), based on the normalised data, they were not included in the analysis. The genes selected were JAK1, JAK2, JAK3, TYK2, STAT1, STAT2,

STAT3, STAT4, STAT5A, STAT5B, and SOCS3. The expression of each was compared between stricture (S) versus no stricture (proximal (P) and distal (D) margin combined), to analyse if there was any differential expression in these segments of the intestine. The cohort of patients is the same as for the bulk sequencing. Demographics and disease phenotype are given in **Table 4-2**. Once analysed, the results were expressed in the form of a heatmap and box plots to get more details on the degree of expression in each segment. These results were correlated with clinical outcomes.

Results of analysis

In our cohort we noted that gene expression of JAK1, STAT1, STAT2, STAT3 were much higher in the stricture compared to non-stricture segments. **(Figure 4-16)** The expression of STAT5A, STAT5B, STAT6 were marginally more expressed in the stricture and there was largely no difference in expression of JAK2, JAK3, TYK2 & STAT4 between stricture and non-stricture. SOCS3 was the only gene in the SOCS family that was expressed in our patient cohort. We noted that it had higher expression in the stricture compared to no stricture. This is illustrated in the heatmap below **(Figure 4-17)**. The heatmap includes all genes collectively selected for the targets of interest. Of these the JAK-STAT pathway is mainly represented by the expression of genes listed above.



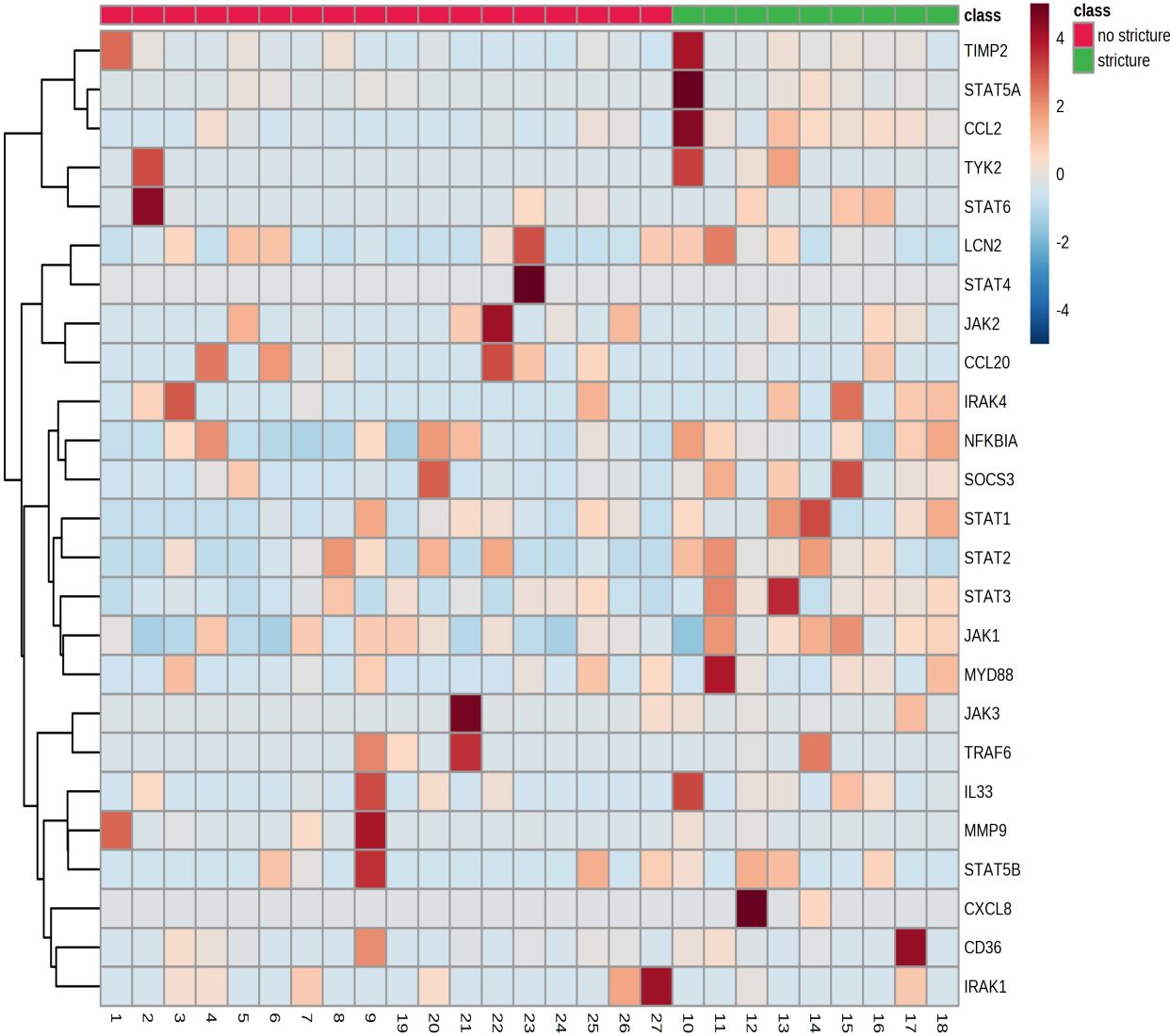


Figure 4-17 Heat map of gene expression of selective genes and pathways

Discussion on our findings

Firstly, our finding that there is differential expression of some JAK-STAT pathway genes in the stricture compared with non-stricture is an important one, highlighting the ongoing disease activity within this segment. This is in line with our findings that CI remains within the microenvironment of the bowel, even in the presence of fibrosis. It is also important to note that

most of the JAK-STAT family was not upregulated in the non-stricture (P+D), except a couple of outliers. This would indicate most patients had a non-inflamed margin at resection.

Secondly, it is interesting to note that JAK1 was the most upregulated in the stricture compared to other members of the family. Similarly, only STAT1, STAT2 and STAT3 were mainly upregulated in the stricture. This probably indicates very specific downstream pathways to be involved in CI and fibrosis in our cohort of patients. JAK1 is a target of therapy already in use in IBD. Selective JAK1 inhibitors have shown promising results in clinical trials. Whether in patients with fibrosis, JAK1 inhibitors will also act in delivering an anti-fibrotic effect is something to be considered. Although STAT5A, STAT5B and STAT6 were marginally upregulated, the difference did not appear to be significant.

Thirdly, JAK2, JAK3 and STAT4 showed no differences in expression between stricture and non-stricture. It is possible that this is indicative of the more dominant pathways at this stage of disease. It was also noted that the dominant negative regulator SOCS3 was upregulated in the stricture compared to non-stricture.

Lastly, our findings pertaining to expression of the STAT family members is also noteworthy. STAT1, STAT2, STAT3 were upregulated, STAT5A, STAT5B, STAT6 were equivocal and STAT4 was not significant. STAT4 is not commonly known to be involved in IBD. It is more active in pathogenesis of RA and Systemic Lupus Erythematosus (SLE). This finding is perhaps not completely unexpected. The upregulation of the other family members again indicates the possibility of CI and their potential for being targets of therapy.

JAK1

JAK1 is widely expressed in tissues, and it is activated by phosphorylation triggered by multiple cytokines - IL-2, IL-4, IL-7, IL-9, IL-15, interferons, IL-10, and growth factors- granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). It has an important role in immune regulation. (Hu, li et al. 2021, Xue, Yao et al. 2023)

STAT1, STAT2, STAT3

These members of the STAT family largely fulfil similar functions and involved in cell differentiation, immune regulation, apoptosis and tumour surveillance.

Together, they can signal via a canonical and non-canonical pathways and have several positive and negative regulators. Targeting STATs may be more specific in achieving therapeutic effect whilst reducing the chances of off-target effects but this is known to be challenging. (Hu, li et al. 2021)

Possible implications from our data and potential applications

In our cohort we report differences in gene expression of the JAK-STAT pathway between the stricture and margins of resection samples. This requires further work looking into quantitative assessments of the genes of interest. It is likely that our findings represent active inflammation within a fibrotic segment which again brings up many points of discussion.

JAK1 was the most upregulated which makes it possible to put forward a hypothesis that JAK1 selective inhibitors may be effective in treating this inflammation within the stricture. Also, it will need further interrogation whether JAK1 inhibitors have stand-alone anti fibrotic properties in addition to an anti-inflammatory role. All patients in our cohort had been exposed to anti-TNF

therapy, which may have suppressed TNF-alpha related inflammation in the stricture. JAK1 upregulation in this location raises the possibility that adding a JAK1 inhibitor to anti-TNFs may have a synergistic effect along with TNF-alpha suppression. This may result in better clinical outcomes. Small molecule oral medications may have better penetration within the stricture and give additional benefits. JAKinibs have been successful as both anti-inflammatory and anti-cancer therapies. In particular JAK1 inhibitors have been more successful at least in clinical trials in UC, compared to pan-JAK inhibitors. (Danese, Vermeire et al. 2022) They may well be effective in B2 phenotype of CD. Future trials may answer this question.

Other members such as TYK2 were not dominant in our dataset. Although there are several TYK inhibitors in clinical trials for fibrosis of other organs systems (Qu, Huang et al. 2016) (Wollin, Maillet et al. 2014), none have been promising in IBD. Our gene expression data is consistent with this observation. Pan-JAK inhibitors have also been tested but were not approved due to significant adverse events. (Huo, Guo et al. 2022) The adverse events could be due to the many off-target effects of non-selective inhibition by first-generation JAKinibs.

Another possible application based on our dataset is targeting of STAT family members. STAT inhibitors have been developed for some time now, as they have an apparent advantage of being more specific downstream inhibition with fewer side effects. However, they have been challenging to implement. Among the STAT inhibitors STAT3 inhibitors are most common and being investigated in clinical trials currently. They have shown some promise in treatment of cancers. (Hu, li et al. 2021) Other STAT family inhibitors are mostly natural compounds (such as curcumin, resveratrol, oleonolic acid), some of which are being investigated in clinical trials for various autoimmune conditions. (Hu, li et al. 2021)

Future work

Our analysis of the pre-selected JAK-STAT pathway gave some interesting results. The next stage of our study was to confirm these findings. Unfortunately, the stage of validating our findings could not be undertaken due to the disruption caused by the pandemic in 2020. The quantitative PCR (q-PCR) analysis of samples was planned for targets found to be significant and interesting, but not all of the planned work could be executed.

We plan to carry out this work in the future to evaluate the merits of our findings. If found promising, JAK1 inhibitors and STAT inhibitors could be evaluated for their anti-fibrotic potential.

4.8 Immunohistochemistry of intestinal strictures

Background

There are novel cytokines that have been reported in recent literature believed to be relevant in inflammation and fibrosis in IBD. As we had full thickness resection tissue, we wanted to explore selected cytokines and investigate their relevance in strictures and resection margins. Some of the most recent reported cytokines include IL-33 and IL-36. We planned to study these cytokines using immunohistochemistry.

Immunohistochemistry (IHC) allows the detection of proteins within tissues and cells and used to determine the expression of the cytokines and chemokines investigated. IHC is a reliable and trusted technique to study targets and cells of interest embedded in tissue. In our cohort, IHC was carried out to look for expression of -

- IL-33 (selected as it is novel and reported to have dual functions)
- IL-36gamma (selected as it could be a potential target of therapy in near future)
- TNFalpha (selected as it is ubiquitous and established in IBD)

Each of these have been discussed in detail below in separate sections.

Patients and methods

We used biomaterial from the same cohort of patients we recruited for the TRAFIC study. Among these patients, 9 were randomly selected for deep immunophenotyping and transcriptomic work. Out of these, 8 patients were finally included for IHC based on the volume of tissue available to continue our work reliably. The demographics of patients that we finally included are given below in **Table 4-3**.

Table 4-3 Demographics and disease classification of patients included for IHC

Patient Study code	Diagnosis duration	Montreal Classification for age -CD	Montreal Classification for location-CD	Montreal Classification for behaviour
BRC-FCD-004	>20 years	A2 between 17 and 40 y	L3 ileocolonic	B2 stricturing
BRC-FCD-016	6 to 12 months	A2 between 17 and 40 y	L3 ileocolonic	B3 penetrating
BRC-FCD-017	10 to 20 years	A2 between 17 and 40 y	L1 ileal	B2 stricturing
BRC-FCD-019	10 to 20 years	A1 below 16 y	L3 ileocolonic	B3 penetrating
BRC-FCD-020	10 to 20 years	A1 below 16 y	L3 ileocolonic	B3 penetrating p perianal disease modifier
BRC-FCD-022	5 to 9 years	A2 between 17 and 40 y	L3 ileocolonic	B2 stricturing
BRC-FCD-023	5 to 9 years	A2 between 17 and 40 y	L1 ileal	B3 penetrating
BRC-FCD-024	5 to 9 years	A2 between 17 and 40 y	L3 ileocolonic	B2 stricturing

Methods for immunohistochemistry

Immunostaining was collaborative work which was led by Dr. Gary Reynolds, a senior staff member in the CLGR labs. Dr. Reynolds is a very experienced scientist and well versed in the technique of IHC as he has a sub-specialty interest in it.

Materials

Tissue samples used

All tissue samples used in these studies were from patients undergoing surgery for stricturing CD. The tissue obtained were formalin fixed and then placed on a tissue processor and taken through to paraffin wax. Following embedding in paraffin wax, tissue blocks were sections at 4microm on

a rotary microtome, placed onto charged slides (PFM, UK) and heated in a 60°C incubator for 1 hour prior to use.

Immunohistochemistry

Pre-treatment of sections

Prior to IHC, all tissue sections were de-waxed in xylene for 5 minutes, placed into alcohol for 5 minutes and then into water for 5 minutes to rehydrate.

Antigen retrieval was performed on tissue sections using the ALTER (Agitated Low Temperature Epitope Retrieval) technique. This comprised of 1 litre of 1mM EDTA pH 8.0/ 0.1% Tween 20 placed into a 2 litre pyrex container and heated on a hot plate stirrer with temperature probe (Jenway 1103; Fisher Scientific), to a temperature of 65°C. Sections were placed into a metal rack and immersed in the buffer overnight (16 hours). During this period the solution was agitated using a 30x5mm magnet bar, with the stirrer set to 600rpm. The container was removed from the hot plate and tap water run into the buffer until room temperature had been reached.

Immunostaining

Following antigen retrieval, slides were placed onto a Dako Autostainer, for immunohistochemistry. Firstly, antibodies required optimisation; the mouse anti-TNF alpha (sc52746, Santa Cruz), had been used in a previous study and so the dilution was known. The rabbit anti-IL-36g (10124-R013, Sino Biologicals Inc) and mouse anti-IL-33 (10368-MM13, Sino Biological Inc) were optimised on 1 case that had responded well and 1 case that had not; antibodies were double diluted using ranges of 1/500 – 1/8000 and 1/25 = 1/400 respectively. Immunostaining on the Autostainer comprised of a 10 minute endogenous peroxidase block in

Boxall (Vector Labs) followed by a 10 minute protein block in 2% casein (Vector Labs). Sections were incubated in primary antibody for one hour; anti-TNF alpha, anti-IL36g, 1/4000 and anti-IL-33, 1/50 dilutions. Antibody detection was performed with Vector Excel mouse and rabbit kits respectively and visualised in NovaRED or impact DAB chromagen (Vector Labs), for 5 minutes followed by a wash in water. All buffer washes performed were with EnVision FLEX wash buffer (Dako).

Following the final water wash, sections were removed from the autostainer and counterstained in Mayers Haematoxylin for 5 minutes. After another wash in water, tissue sections were dehydrated in absolute alcohol, 2 x2 minute washes, placed into xylene for 5 minutes and then mounted with glass coverslip in a permanent resin based mounting medium, DPX (CellPath).

Analysis

All immunostaining was then evaluated microscopically. A comprehensive yet user-friendly scoring system was developed for this purpose to easily describe the findings in a quantified format. Dr. Reynolds proposed this, and we finally used an Expression Score (ES) system. As per this system we described the immunostaining as negative (0), low (1-2), medium (3-4) or high (5-6). This was determined by assessment of percentage of inflammatory cells staining: >50%, 3; 25-50%, 2; <25% 1; and stain intensity: strong, 3; moderate, 2; weak, 1. Assessment was split into mucosa and submucosa to see whether any differences were observed and then combined as an overall total (Expression Score). Where mucosa or submucosa were absent, just the one score was used for the ES. Also, all negative stains were repeated to ensure this was a true representation of expression of the cytokine.

Examples of high, moderate, and low ES for each of the cytokines are given in **Figures 4-18, 4-19 and 4-20.**

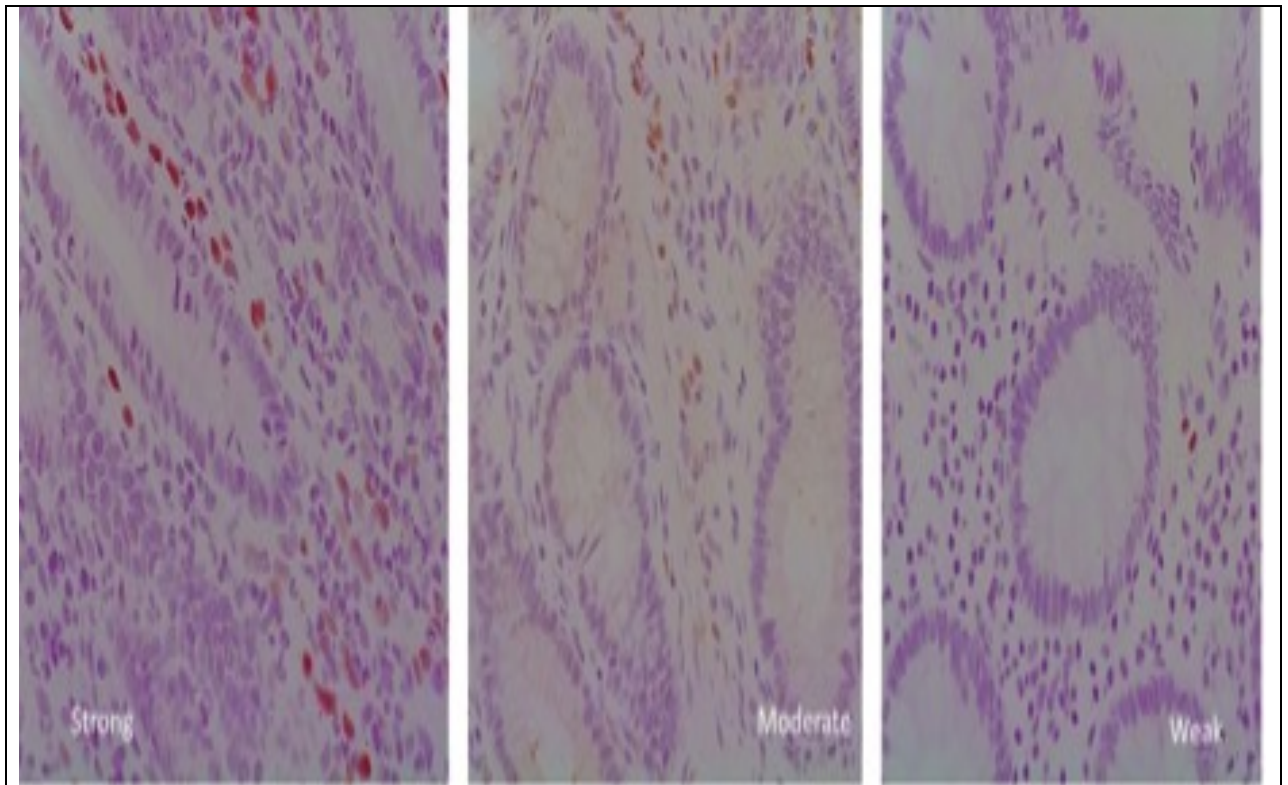
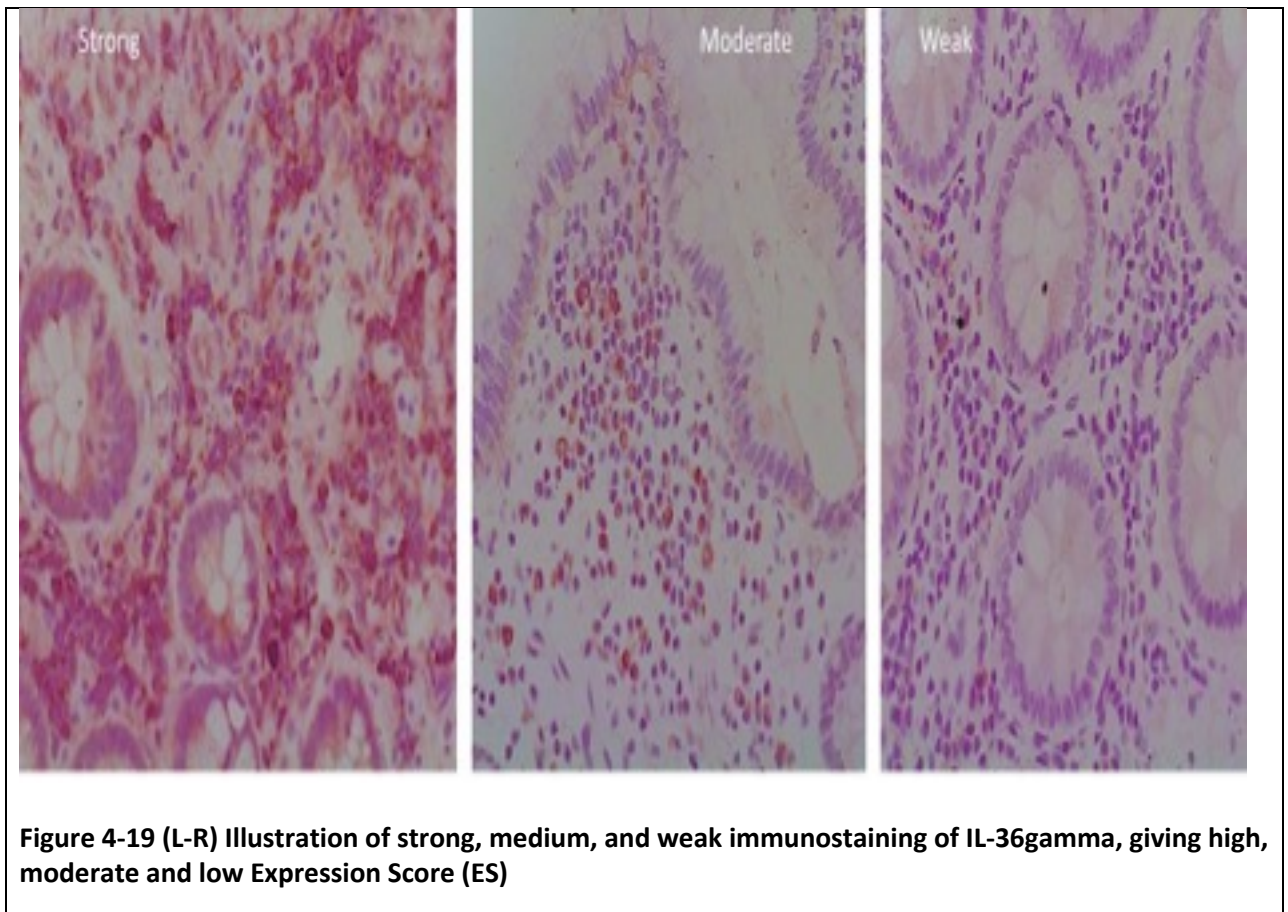


Figure 4-18 (L-R) Illustration of strong, medium and weak immunostaining of IL-33, giving high, moderate and low Expression Score (ES)



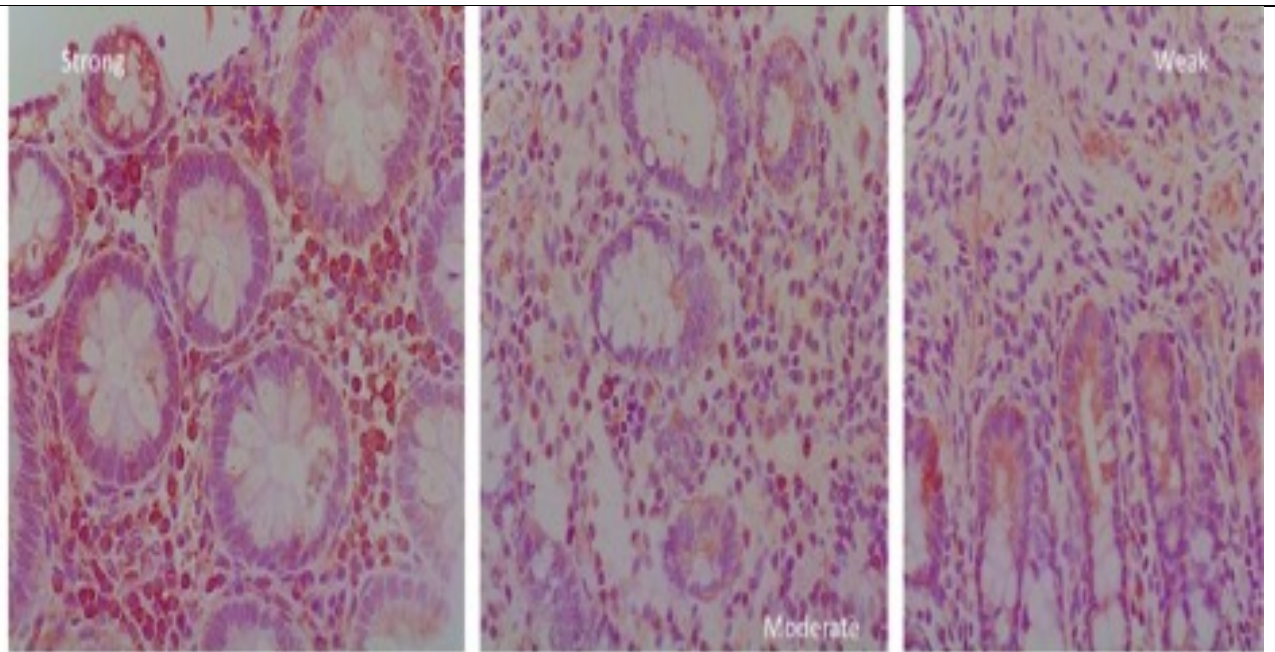


Figure 4-20 (L-R) Illustration of strong, medium, and weak immunostaining of TNFalpha, giving high, moderate, and low Expression Score (ES)

The immunostaining analysis gave us some interesting results and findings. Some select examples of patients only have been discussed in separate sections for each cytokine of interest. Data from other patients is available with us for future discussion.

4.8.1 Interleukin-33

Interleukin-33 (IL-33) is a member of the IL-1 family. This group of cytokines includes IL-1 α , IL-1 β , and IL-18, that act to initiate inflammatory responses secondary to local stress.(Schmitz, Owyang et al. 2005) IL-33 interestingly, behaves as both an extracellular, traditional cytokine as well as a nuclear transcription factor.(Ali, Mohs et al. 2011, Hodzic, Schill et al. 2017) IL-33 is a relatively new discovery first reported in 2005.(Schmitz, Owyang et al. 2005) Initially, this cytokine was recognised as a potent activator integral to adaptive immunity,(Hodzic, Schill et al. 2017) but with increasing understanding, it is now known to be active as a key player in both innate and adaptive immunity. It exerts action by binding to its receptor suppression of tumorigenicity 2 (ST2). ST2 has been shown to modulate both innate & adaptive immune systems and expressed by several important immune cells. This mainly includes T helper 1 (T_H1) cells, T_H2 cells, group 2 innate lymphoid cells (ILC2s), regulatory T (Treg) cells, and CD8⁺ T cells.(Hodzic, Schill et al. 2017)

IL-33 has generated a lot of interest recently due to its diversity of functions, and implications in various physiological and pathological contexts. It is primarily produced by stromal and epithelial cells as well as immune cells such as dendritic cells and macrophages, acting as a nuclear alarmin when released upon cellular stress or damage. Its receptor, ST2, exists in both membrane-bound and soluble forms, allowing for pleiotropic signalling pathways. The IL-33/ST2 axis plays a pivotal role in maintaining immune homeostasis and driving various immune processes. It has multiple functions which continue to be a subject of ongoing research.

One of the most important functions of IL-33 is immunomodulation. It has a prominent role in shaping the immune response. It acts as an alarmin and stimulates both innate and adaptive immune cells, including mast cells, eosinophils, Th2 cells, regulatory T cells, and natural killer cells.

It has been shown to activate Th2 cells and induce the production of cytokines such as interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13). (Cayrol and Girard 2022) By promoting Th2 responses and inhibiting Th1 and Th17 pathways, IL-33 plays a crucial role in the regulation of allergic responses, autoimmune diseases, and chronic inflammation.

Several studies have highlighted the involvement of IL-33 in various chronic inflammatory diseases, such as asthma, atopic dermatitis, rheumatoid arthritis, and IBD. It is also implicated in neuro-inflammation and pathogenesis of neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis. Its ability to amplify inflammation makes it a potential therapeutic target to control inflammation. It has been reported as a potential target of therapy in IBD too with better understanding of its role in IBD patients. (Dubois-Camacho, Diaz-Jimenez et al. 2019)

Another important role it has is in tissue repair and regeneration. It promotes the activation and differentiation of fibroblasts, thus contributing to wound healing and tissue remodelling. However, the dysregulation of IL-33 signalling can lead to excessive fibrosis, potentially exacerbating organ damage. (Li, Guabiraba et al. 2014)

The intricate involvement of IL-33 in various diseases has sparked interest in its therapeutic potential. Several preclinical studies have explored the modulation of IL-33 signalling as a treatment strategy for inflammatory disorders, cancer immunotherapy, and tissue regeneration. But it has dual functions and probably have a protective effect in some chronic inflammatory conditions. (Tahaghoghi-Hajghorbani, Ajami et al. 2019) Blocking IL-33 or its receptor has shown promise in preclinical (Waddell, Vallance et al. 2015) and some early clinical studies for the treatment of allergic diseases and autoimmune conditions. Conversely, the administration of IL-33 or its agonists may be beneficial in stimulating immune responses against cancer as it has been shown to have some anti-tumorigenic properties. (Lu, Yang et al. 2016) Before it is taken from

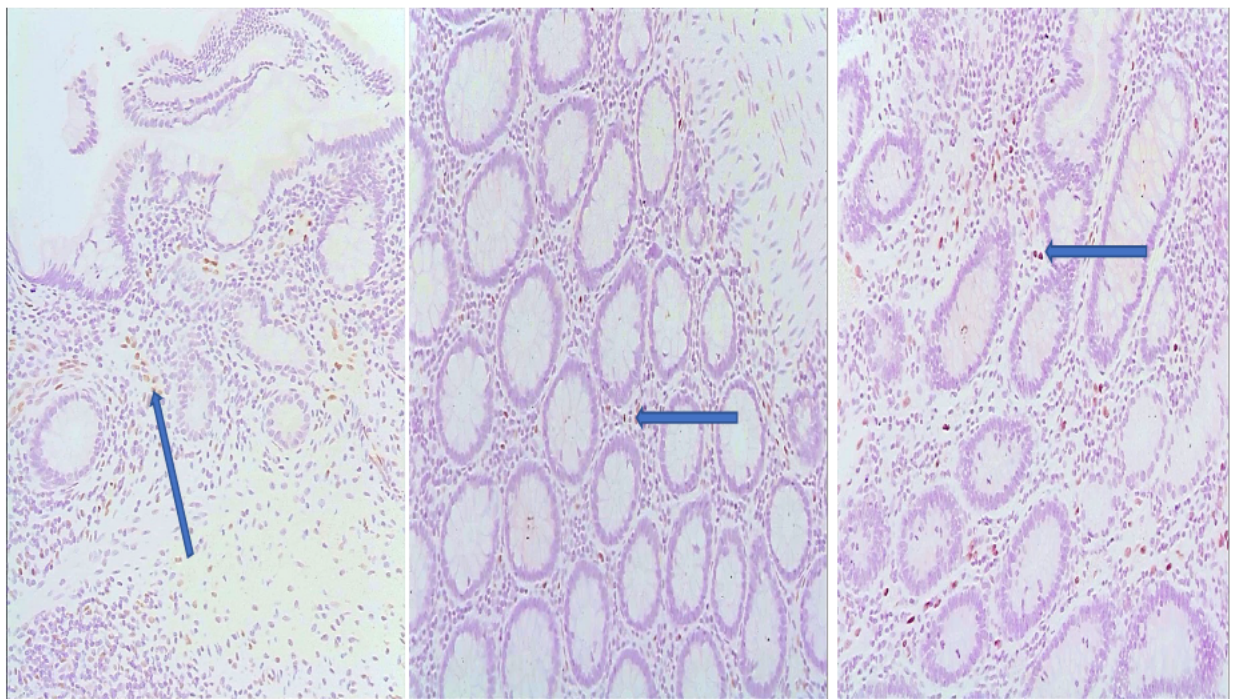
bench to bedside, a thorough understanding is necessary to ensure safety. Due to its dual function, IL-33 remains of interest and subject of ongoing research. We wanted to explore this cytokine in more detail and its role in CD related strictures, using IHC techniques.

Results of immunostaining in select patients

All of the 8 patients in whom IHC was undertaken were analysed in detail. The findings on IHC were correlated with clinical outcomes. 3 patients were selected for discussion in this thesis intended to cover a range of clinical outcomes. (Very poor, Moderate and Good outcomes).

The images of IHC for these patients are presented below. The basic demographics, biologics exposure status and clinical outcomes are given for each of these patients.

Patient BRC-FCD-004



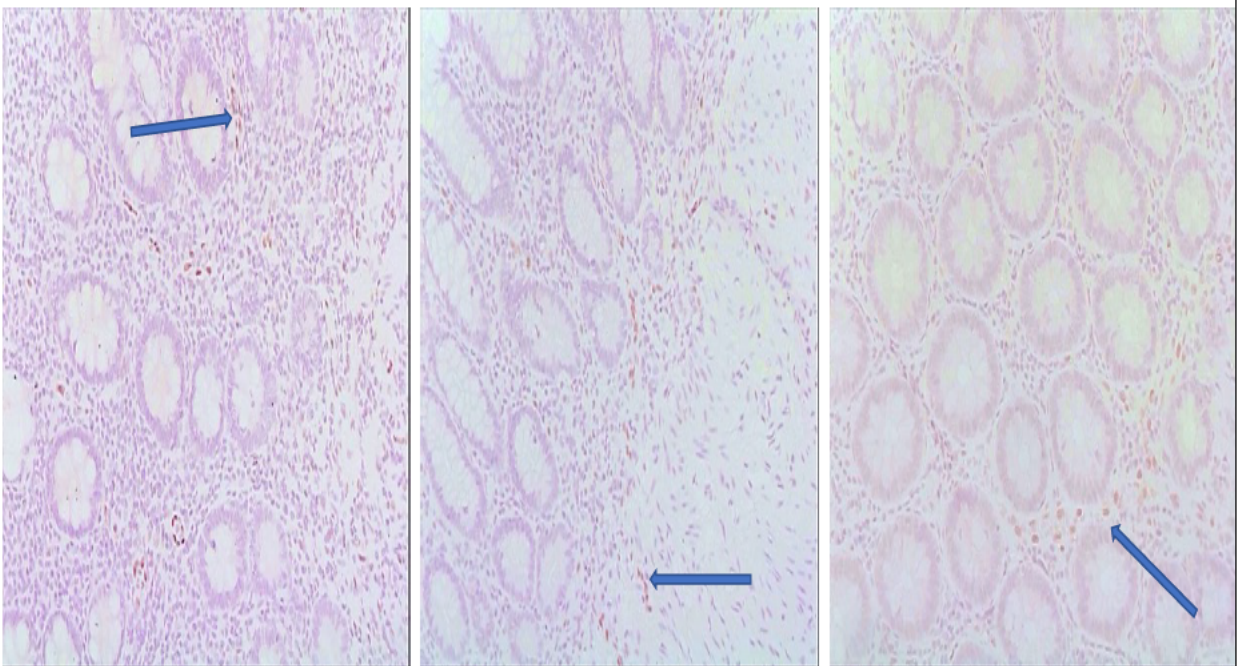
(L-R) – This slide for patient FCD-004 illustrates immunostaining of IL-33 (arrows) in the Stricture (S), Proximal (P) and Distal (D), respectively.

Figure 4-21 FCD-004 IHC slide with high ES across all 3 segments

	Stricture			Non-stricture				
Patient code	Biologic before surgery	Stricture IL 33 ES M	Stricture IL 33 ES SM	Proximal IL 33 ES M	Proximal IL 33 ES SM	Distal IL 33 ES M	Distal IL 33 ES SM	Outcome
BRC-FCD-004	Anti TNF	5	6	5	6	5	6	Very poor
Overall score			High		High		High	

Patient FCD-004 had a disease classification of A2L3B2. Patient had high ES in all 3 segments and The patient had a very poor outcome due to recurrence of disease within 6 months of surgery requiring escalation to another class of biologics.

Patient BRC-FCD-016



(L-R) – This slide for patient FCD-016 illustrates immunostaining of IL-33 (arrows) in the Stricture (S), Proximal (P) and Distal (D), respectively

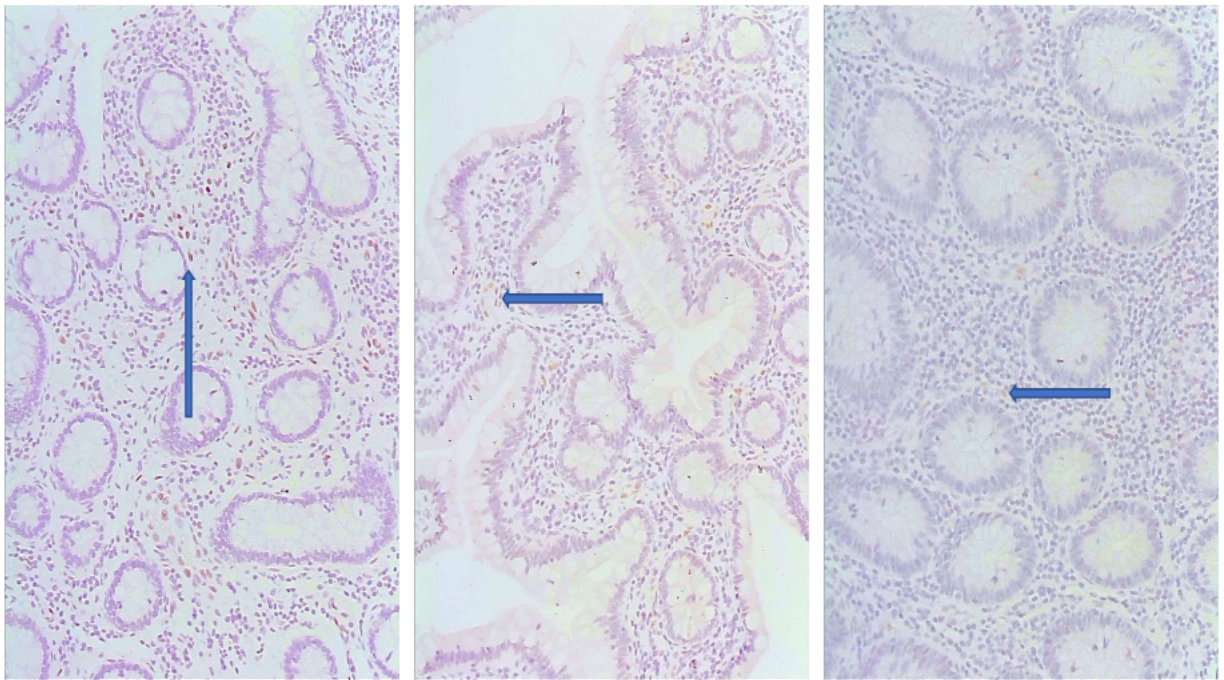
Figure 4-22 FCD-016 IHC slide with high ES in stricture and proximal segment and moderate expression in the distal segment

Patient FCD-016 had a disease classification of A2L3B3. Patient had high ES in 2 out of 3 segments.

The distal margin had moderate expression of IL-33. The patient went on to have a good outcome and no AOs were recorded until June 2023.

Patient code	Biologic before surgery	Stricture		Non-stricture				Outcome
		Stricture IL 33 ES M	Stricture IL 33 ES SM	Proximal IL 33 ES M	Proximal IL 33 ES SM	Distal IL 33 ES M	Distal IL 33 ES SM	
BRC-FCD-016	Anti TNF	5	5	6	6	3	4	Good
Overall score			High		High		Moderate	

Patient BRC-FCD-020



(L-R) – This slide for patient FCD-020 illustrates immunostaining of IL-33 (arrows) in the Stricture (S), Proximal (P) and Distal (D), respectively

Figure 4-23 FCD-020 IHC slide with high ES in stricture and moderate ES in the proximal and distal segments

Patient FCD-020 had a disease classification of A1L3B3. Patient had a high ES in the stricture and moderate ES in the proximal and distal margins.

The patient had a poor outcome with flare of disease requiring escalation to biologics after 6 months of surgery.

Patient code	Biologic before surgery	Stricture		Non-stricture				Outcome
		Stricture IL 33 ES M	Stricture IL 33 ES SM	Proximal IL 33 ES M	Proximal IL 33 ES SM	Distal IL 33 ES M	Distal IL 33 ES SM	
BRC-FCD-020	Anti TNF	5	5	4	4	3	3	Poor
Overall score			High		Moderate		Moderate	

For ease of understanding and flow, the discussion of findings for each selected cytokine has been presented separately in respective sections. A collective discussion around IHC and the overall conclusions have also been presented at the end of the chapter.

Discussion of IHC in IL-33

The experiments using IHC on the resected tissue brought some interesting results worthy of further discussion and work. They are addressed here broadly based on the expression levels of the cytokine and segment (location) of bowel in which it is expressed.

Expression Score within strictures

In patients selected to cover the range of clinical outcomes, we noted that the expression of IL-33 in the stricture was high in all patients, as per the overall ES. The high expression did not appear to have any influence on the clinical outcome of the patients. It is possible that high expression in the stricture is secondary to sub-optimal control of the inflammatory cascade that has led to

secretion of IL-33. IL-33 is known to have a proinflammatory effect in IBD. (Pastorelli, De Salvo et al. 2013) Its presence may have also contributed to fibrosis. Similar fibrogenesis potential of IL-33 been reported in other organ systems. (Li, Guabiraba et al. 2014)

Another possible explanation for abundant presence of IL-33 could be do with fibroblast population in the strictures. It has been reported in some preclinical models that IL-33 is secreted by fibroblasts (Waddell, Vallance et al. 2021, Aggeletopoulou, Tsounis et al. 2023). However, what effects it has later remains unclear, due to its dual nature.

Expression Score in margins (proximal and distal segments)

We noted that the high expression of IL-33 in the either the proximal or distal margins did not have any bearing on the clinical outcomes. It is worth noting that patient FCD-004 who had high ES in both margins went on to have a poor outcome, probably indicating there was microscopic inflammation in margins. However, this was not a consistent finding and difficult to draw any inference from.

Expression Scores in strictures and margins

An observation of collective ES across all segments also did not have any obvious influence on clinical outcomes. It is possible that the high degree of inflammation in the stricture could be main driver of inflammation within that microenvironment. The resection of this inflammatory burden may itself be sufficient to reduce the risk of disease recurrence, despite the observation that some of the margins showed high ES, indicating microscopic inflammation. This is perhaps an indication that IL-33 does indeed have dual functions depending on the stage and age of inflammation as shown in other studies. (Waddell, Vallance et al. 2015, Waddell, Vallance et al. 2021)

4.8.2 Interleukin-36-gamma

IL-36-gamma (IL-36g), originally known as IL-1F9, has emerged as a critical regulator of inflammatory processes, modulating immune cell activation, tissue remodelling, and cytokine production. It shares structural similarities with other IL-1 family members. It signals through a receptor complex comprising IL-36R (IL-36 receptor) & IL-1RAcP, and plays a substantial role in shaping host immunity by exerting influence on both innate and adaptive arms of the immune system. (Ngo, Kuczma et al. 2021) Functionally, IL-36 α /IL-36 β /IL-36g act as IL-36 receptor agonists. IL-36g-receptor complex triggers downstream signalling cascades involving the nuclear factor kappa B (NF- κ B), mitogen-activated protein kinases (MAPKs), and caspase-1, leading to the production of pro-inflammatory cytokines and chemokines. (Ngo, Kuczma et al. 2021) It has been reported that it can signal via the STAT transcription pathway and influence both Treg and Th9 cells. (Harusato, Abo et al. 2017) Studies have shown that IL-36 receptor agonists can affect a wide range of immune cells such as dendritic cells, macrophages, T cells, keratinocytes and myofibroblasts. (Ngo, Kuczma et al. 2021) It has been implicated in several autoimmune disorders including psoriasis, RA, COPD and renal diseases.

More recently it has been reported that IL-36g play an active role in gut immunity and it appears to be rather complex. It can be both protective, and detrimental. According to reports from murine models of colitis, it has a differential role depending on the time of expression and phase of the disease. IL-36 α and IL-36g expression have been noted to be upregulated in patients with active IBD. (Russell, Horan et al. 2016) The dichotomous roles of IL-36 cytokines for both acute and chronic settings in IBD have also been shown in multiples studies. (Medina-Contreras, Harusato et al. 2016, Scheibe, Backert et al. 2017, Scheibe, Kersten et al. 2019) IL-36g appears to

exert pleiotropic effects on immune cells and various cell types within the tissue microenvironment.

Of note is its role in tissue remodelling and fibrosis. Scheibe and colleagues comprehensively demonstrated increased expression of IL-36a and IL-36g in human IBD tissue. They also reported its key role in activation of fibroblasts, (Scheibe, Backert et al. 2017) and further demonstrated that inhibiting this receptor reduced the occurrence of fibrosis in the intestine. (Scheibe, Kersten et al. 2019)

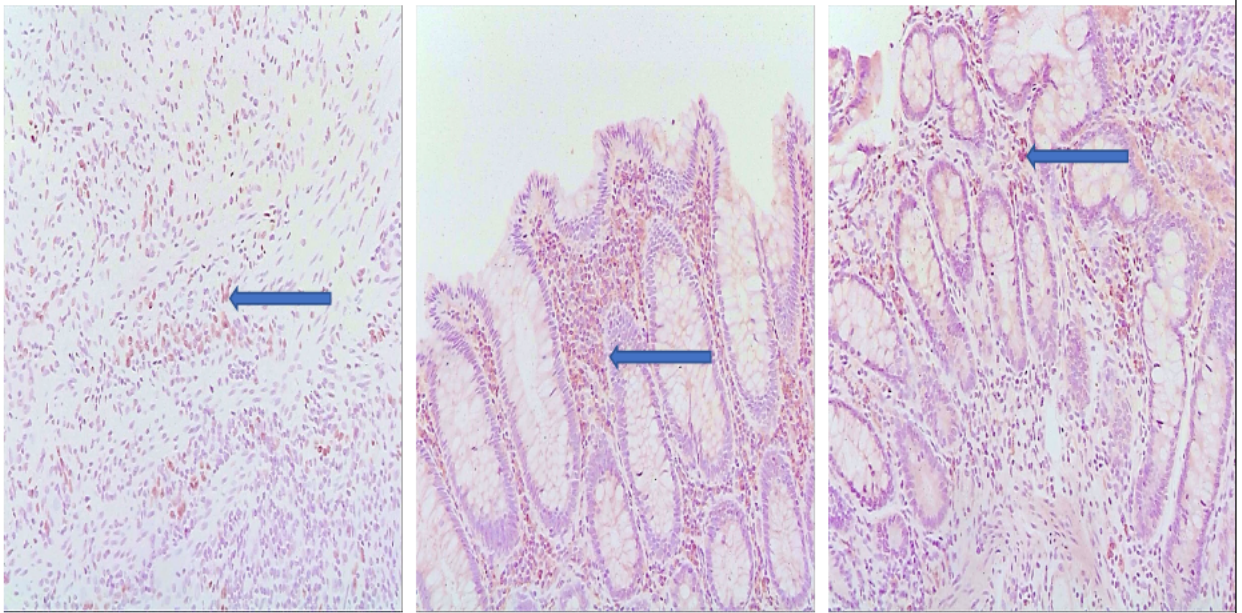
It has no doubt emerged as a target of therapy in autoimmune conditions. Indeed, it has been investigated in clinical trials. A monoclonal antibody against the IL-36 receptor was used to treat a rare form of pustular psoriasis with promising results in a proof of concept study. (Bachelez, Choon et al. 2019) Further trials are awaited. It was of interest in IBD (Mao and Rieder 2019) but so far no formal reports from clinical trials have been published.

We wanted to explore the role of IL-36g in chronic intestinal inflammation & stricturing CD using the biomaterial from our cohort of patients, to better understand its biological functions and examine its potential as a target of therapy.

Results of immunostaining in select patients

As described in the previous section, 8 patients in whom IHC was undertaken were analysed in detail and their findings were correlated with clinical outcomes. 3 patients were selected for discussion in this thesis intended to cover a range of clinical outcomes. (Very poor, Moderate and Good outcomes).

The images of IHC for these patients are presented below. The basic demographics, biologics exposure status and clinical outcomes are given for each of these patients.

Patient BRC-FCD-004

(L-R) – This slide for patient FCD-004 illustrates immunostaining of IL-36g (arrows) in the Stricture (S), Proximal (P) and Distal (D) margins, respectively.

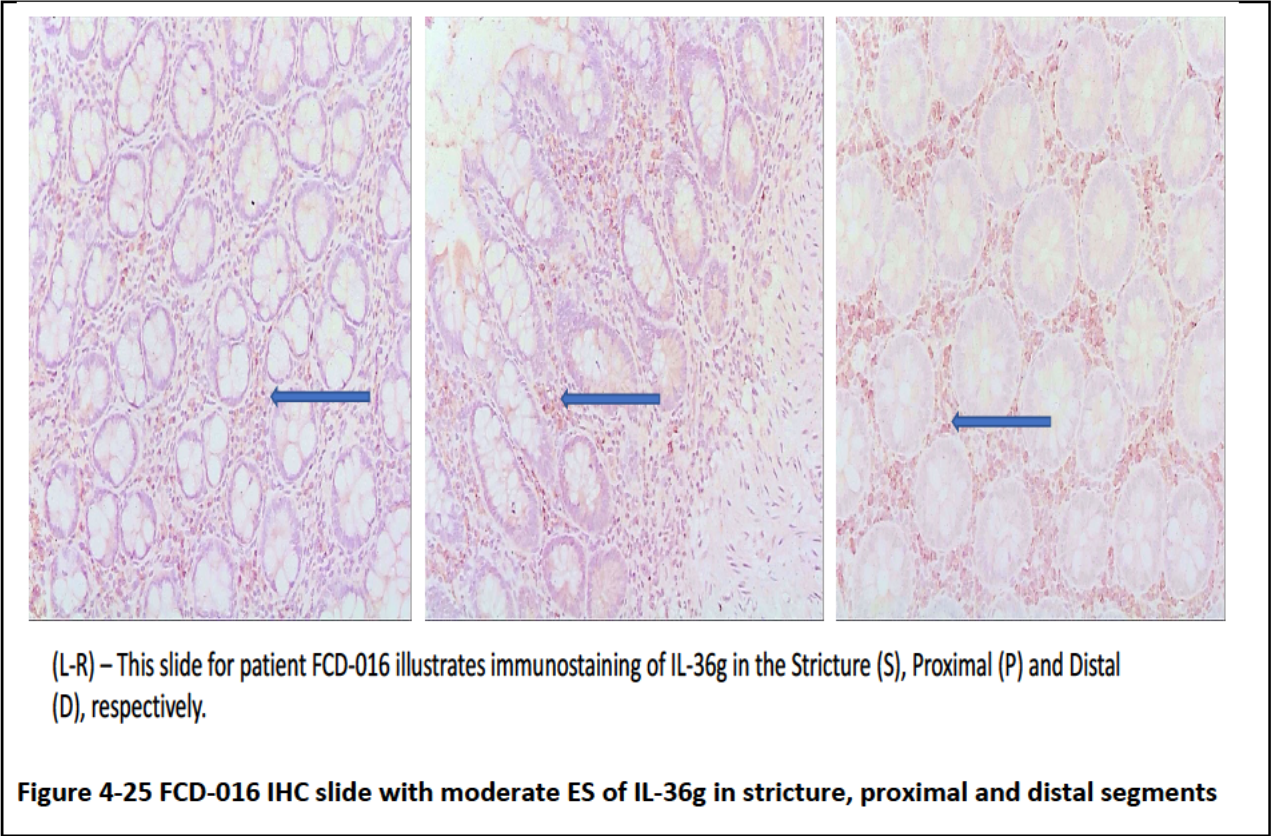
Figure 4-24 FCD-004 IHC slide with high ES of IL36g in stricture and proximal segments, and moderate ES in the distal segment

Patient FCD-004 had a disease classification of A2L3B2. This patient had high expression of IL-36g in the stricture and proximal segments and moderate expression in the distal segment.

The patient had a very poor outcome due to recurrence of disease within 6 months of surgery requiring escalation to another class of biologics.

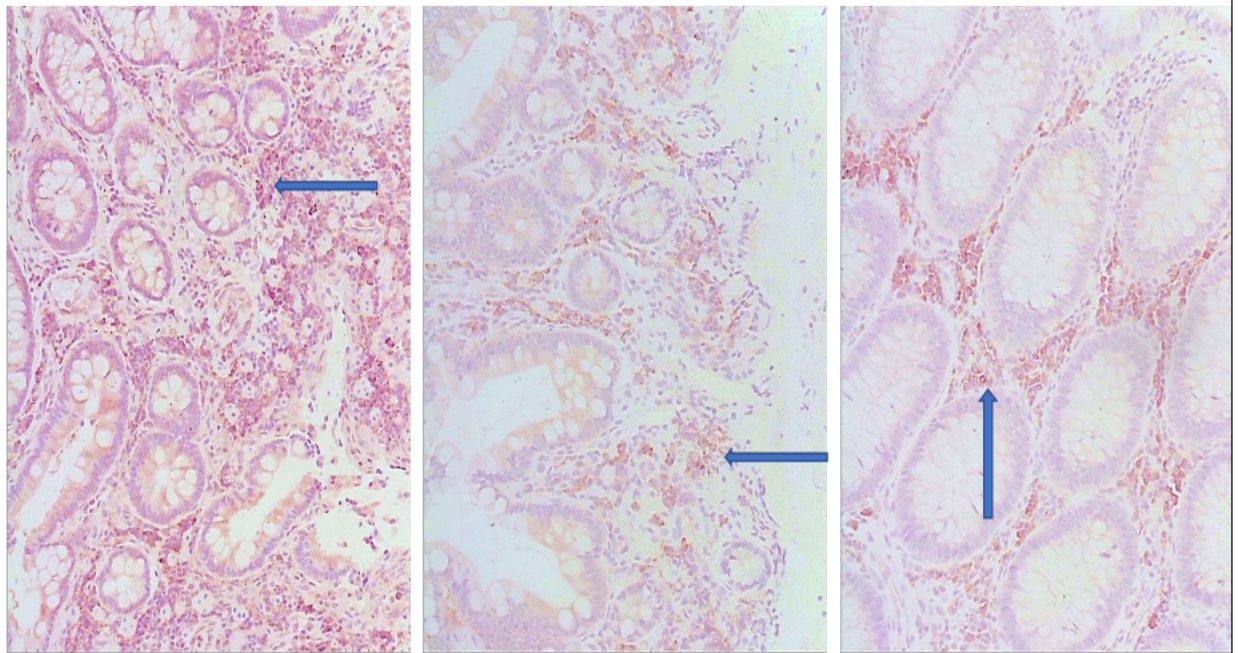
Patient code	Biologic before surgery	Stricture		Non-stricture				Outcome
		Stricture IL 36g ES M	Stricture IL 36g ES SM	Proximal IL 36g ES M	Proximal IL 36g ES SM	Distal IL 36g ES M	Distal IL 36g ES SM	
BRC-FCD-004	Anti TNF	6	4	5	4	3	4	Poor
Overall score			High		High		Mod	

Patient BRC-FCD-016



Patient FCD-016 had a disease classification of A2L3B3. Patient had moderate expression of IL-36g in all 3 segments and went on to have a good outcome with no adverse outcomes recorded at follow up until June 2023, that is more than 3 years after surgery.

Patient code	Biologic before surgery	Stricture		Non-stricture				Outcome
		Stricture IL 36g ES M	Stricture IL 36g ES SM	Proximal IL 36g ES M	Proximal IL 36g ES SM	Distal IL 36g ES M	Distal IL 36g ES SM	
BRC-FCD-016	Anti TNF	3	4	3	5	4	4	Good
Overall score			Mod		Mod		Mod	

Patient BRC-FCD-020

(L-R) – This slide for patient FCD-020 illustrates immunostaining of IL-36g (arrows) in the Stricture (S), Proximal (P) and Distal (D), respectively.

Figure 4-26 FCD-020 IHC slide with high ES of IL-36g in stricture, proximal and distal segments

Patient FCD-020 had a disease classification of A1L3B3. This patient had high expression of IL-36g in all 3 segments. The patient had a poor outcome with flare of disease requiring escalation to biologics after 6 months of surgery.

		Stricture		Non-stricture				
Patient code	Biologic before surgery	Stricture IL 36g ES M	Stricture IL 36g ES SM	Proximal IL 36g ES M	Proximal IL 36g ES SM	Distal IL 36g ES M	Distal IL 36g ES SM	Outcome
BRC-FCD-020	Anti TNF	6	5	5	4	5	5	Poor
Overall score			High		High		High	

Discussion of IHC for IL-36g

In our cohort, we found expression of IL-36g in the intestine to be high in most segments and moderate in some. The possible reasons are discussed below, and the implications are discussed in a later section collectively. The points of discussion are addressed here broadly based on the expression levels of the cytokine and the segment (location) of bowel in which it is expressed.

Expression Score within strictures

In patients selected to cover the range of clinical outcomes, we noted that the expression of IL-36g in the stricture was high in 2 patients and moderate in 1 patient, as per the overall ES. We observed that the patients in whom the expression was high within the stricture the outcomes were poor whereas when it was moderate, the outcome was good. Due to the pleiotropic effects of IL-36, it is difficult to ascertain whether this is the cause of inflammation or the effect of fibrosis. IL-36g has been shown to be involved in the mucosal healing process, (Scheibe, Backert et al. 2017) (Boutet, Bart et al. 2016, Medina-Contreras, Harusato et al. 2016) but in more advanced disease, it has a proinflammatory effect and can worsen inflammation. (Russell, Horan et al. 2016) It is also known to induce fibrosis and known to be secreted by adipocytes, (Frühbeck, Gómez-Ambrosi et al. 2022) a cell population part of the stricture microenvironment. Given that our cohort had established disease, they were all in the chronic phase, making it more likely that IL-36g could be exerting a proinflammatory effect. In any of the three possibilities, our findings confirm the role of IL-36g in CD-related strictures, making it a potential target of therapy for this group of patients.

Expression Score in margins (proximal and distal segments)

We noted that the expression of IL-36g in the resection margins possibly has an effect on the outcomes. In FCD-016, the expression levels were low/borderline-moderate in both proximal and distal segments, probably indicating lower levels of inflammation. This patient went on to have a good outcome with no adverse outcomes. When one of the margins had high expression, the outcome was poor. The presence of IL-36g indicates microscopic inflammation which is an important prognostic factor for early recurrence.

Expression Scores in strictures and margins

An observation of collective ES across all segments indicates that higher expression in the margins probably result in poorer outcomes. The patient who had a good outcome had low to moderate ES across all segments. Despite the dual role IL-36g plays in gut immunity, based on the current literature, it would be safe to say the high expression levels within the margins and stricture very likely represent active inflammation.

4.8.3 Tumour Necrosis Factor-alpha (TNF-alpha)

Tumour necrosis factor-alpha (TNF alpha) is a pleiotropic cytokine, which is known to have homeostatic functions in addition to its immune and inflammatory roles.(Kallioliias and Ivashkiv 2016) It is essential for optimal defence against pathogens, development of granuloma formation, resolution of inflammation and induction of tissue repair. Some studies have also described its role in tissue regeneration such as neuronal remyelination, cardiac remodelling and cartilage regeneration. (Kallioliias and Ivashkiv 2016)

The role of TNF alpha as a proinflammatory cytokine is also widely reported in many studies. It has been shown to induce inflammation, activate vascular endothelium, recruit immune cells, and promote tissue destruction. It is well understood that excessive production or function of TNFa has been linked to inflammatory diseases such as IBD, RA, psoriasis, psoriatic arthritis, ankylosing spondylitis among others. Both homeostatic and pathogenic activities of TNFa are mediated by distinct molecular and cellular pathways.

In the context of IBD, TNFa is overproduced and dysregulated, leading to excessive immune activation and the perpetuation of chronic inflammation. It promotes recruitment of immune cells in inflamed intestinal tissue leading to constant tissue damage and the release of additional proinflammatory molecules. TNFa is known to stimulate the production of other pro-inflammatory cytokines, such as IL-1 and IL-6. These cytokines further amplify the inflammatory response and contribute to destruction of the intestinal barrier. More recently, it has also been implicated in fibrosis. Some studies have reported its effects on fibroblasts and myofibroblasts, making it a possible target of therapy. (Verjee, Verhoekx et al. 2013)

Given the central role of TNFa in IBD, therapeutic strategies targeting this cytokine have been developed. Monoclonal antibodies (anti-TNFa) were the first biologic drugs approved for treatment of IBD. (Rutgeerts, Sandborn et al. 2005) Currently, multiple drugs namely infliximab, adalimumab, golimumab and certolizumab pegol, are widely used in the treatment of moderate to severe IBD. Due to their widespread presence as well as multiple immune functions, suppression of anti-TNFa leads to multiple side effects which we manage in clinical practice. (Shivaji, Sharratt et al. 2019)

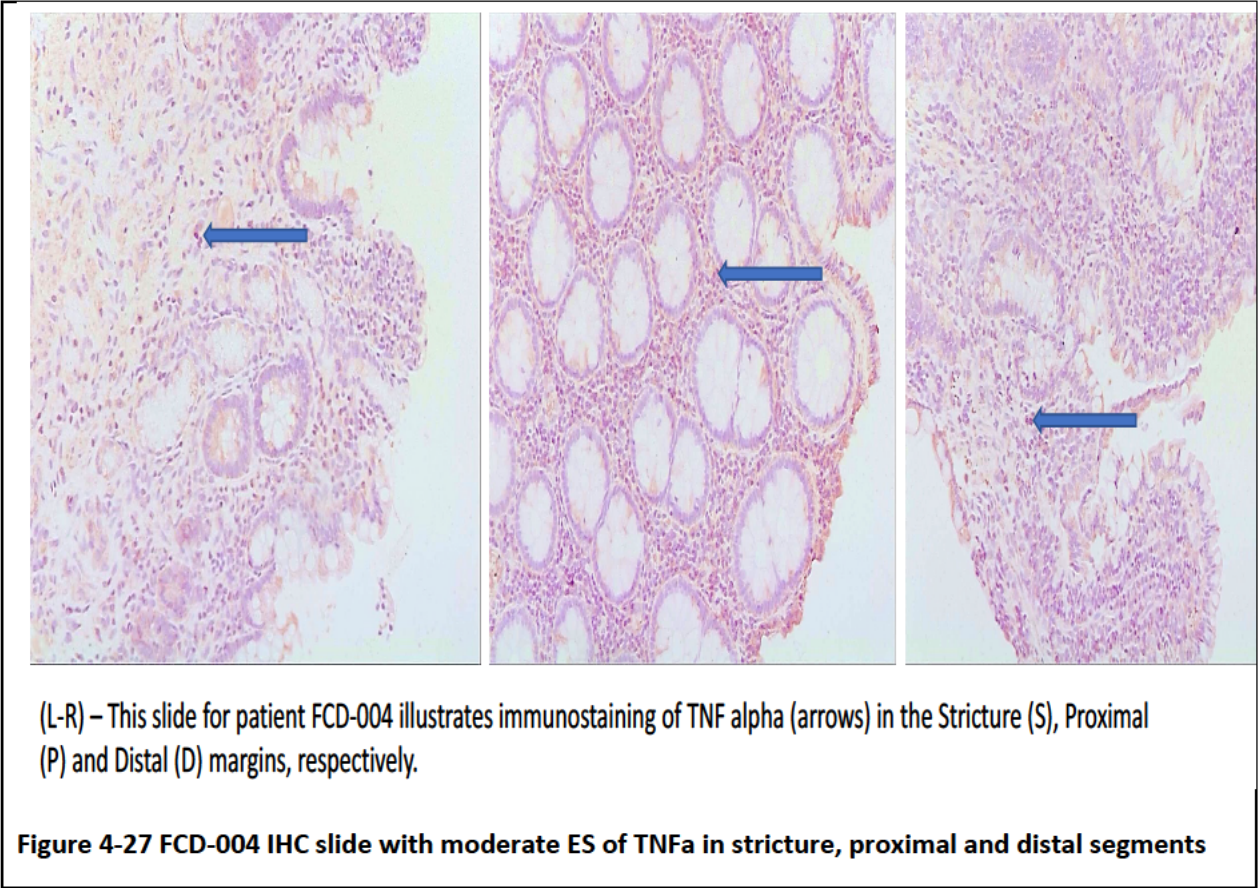
We selected this cytokine for our experiments for a few reasons. TNFa is one of the most widely studied cytokines, ubiquitously present in tissue of IBD patients, making it a reliable marker of inflammation. Anti-TNFa drugs are the most commonly used biologic drugs for treatment of IBD, which clinicians are most familiar with. These characteristics make it a reliable control for IHC experiments on intestinal tissue.

Results of immunostaining in select patients

8 patients in whom IHC was undertaken were analysed in detail and their findings pertaining to TNFa immunostaining were correlated with clinical outcomes. 3 patients were selected for discussion in this thesis intended to cover a range of clinical outcomes. (Very poor, Moderate and Good outcomes).

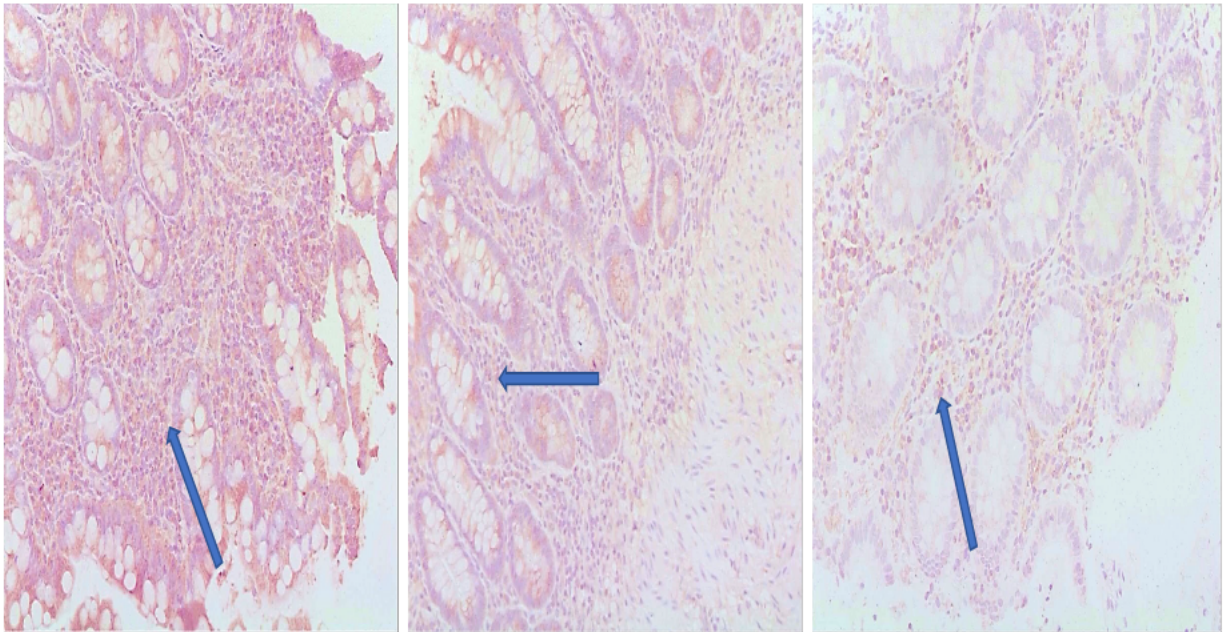
The images of IHC for these patients are presented below. The basic demographics, biologics exposure status and clinical outcomes are given for each of these patients.

Patient BRD-FCD-004



Patient FCD-004 had a disease classification of A2L3B2. Patient had moderate ES of TNF alpha in all 3 segments. The patient had a very poor outcome due to recurrence of disease within 6 months of surgery requiring escalation to another class of biologics.

Patient code	Biologic before surgery	Stricture		Non-stricture				Outcome
		Stricture TNFa ES M	Stricture TNFa ES SM	Proximal TNFa ES M	Proximal TNFa ES SM	Distal TNFa ES M	Distal TNFa ES SM	
BRC-FCD-004	Anti TNF	2	3	2	3	2	3	Poor
Overall score			Mod		Mod		Mod	

Patient BRC-FCD-016

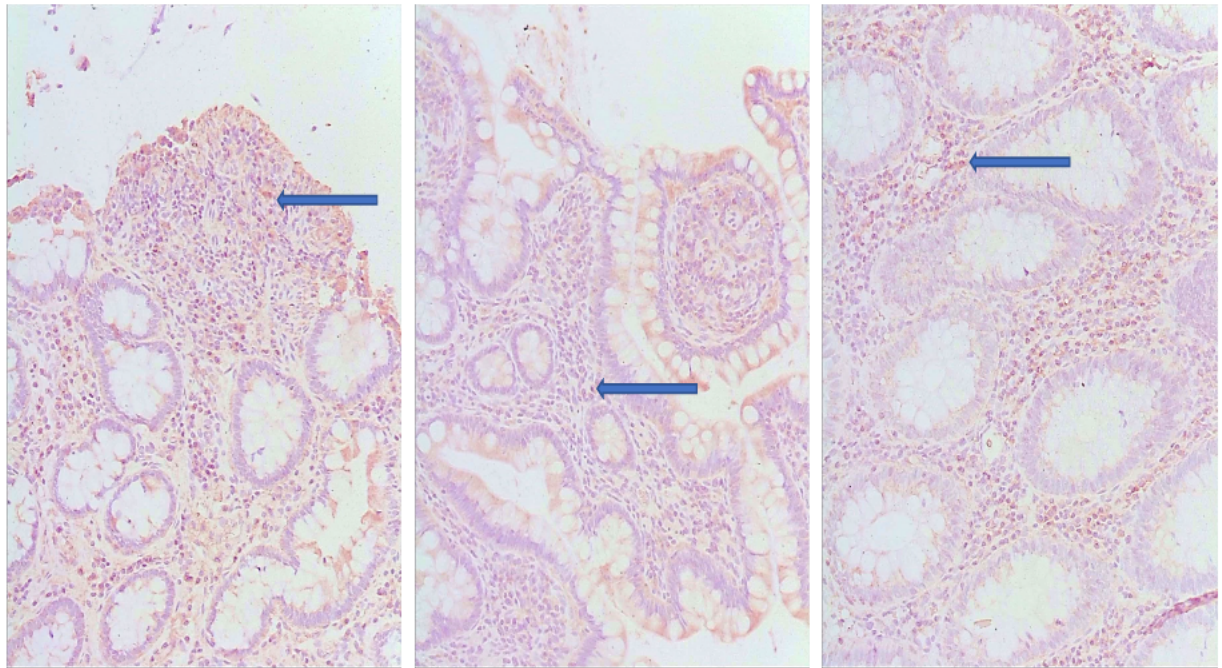
(L-R) – This slide for patient FCD-016 illustrates immunostaining of TNF alpha (arrows) in the Stricture (S), Proximal (P) and Distal (D), respectively.

Figure 4-28 FCD-016 IHC slide with moderate ES of TNFa in stricture, proximal and distal segments

Patient FCD-016 had moderate ES of TNF alpha in all 3 segments. This patient had a good outcome with no adverse outcomes recorded at follow up until June 2023, that is more than 3 years after surgery.

	Stricture			Non-stricture				
Patient code	Biologic before surgery	Stricture TNFa ES M	Stricture TNFa ES SM	Proximal TNFa ES M	Proximal TNFa ES SM	Distal TNFa ES M	Distal TNFa ES SM	Outcome
BRC-FCD-016	Anti TNF	3	2	2	4	4	4	Good
Overall score			Mod		Mod		Mod	

Patient BRC-FCD-020



(L-R) – This slide for patient FCD-020 illustrates immunostaining of TNF alpha in the Stricture (S), Proximal (P) and Distal (D), respectively.

Figure 4-29 FCD-020 IHC slide with moderate ES of TNFa in stricture, low ES in proximal and high ES in the distal segment

Patient FCD-020 had varying ES of TNF alpha in each segment. The expression was moderate in the stricture, low in the proximal and high in the distal segment. This patient had a poor outcome due to recurrence of disease after 6 months of surgery.

Patient code	Biologic before surgery	Stricture		Non-stricture				Outcome
		Stricture TNFa ES M	Stricture TNFa ES SM	Proximal TNFa ES M	Proximal TNFa ES SM	Distal TNFa ES M	Distal TNFa ES SM	
BRC-FCD-020	Anti TNF	4	4	2	2	4	5	Poor
Overall score			Mod		Low		High	

Discussion of IHC for TNF alpha

In our cohort, we found expression of TNF alpha in the intestine to be low or moderate in most segments. The possible reasons are discussed below, and the implications are discussed in a later section collectively. The points of discussion are addressed here broadly based on the expression levels of the cytokine and the segment (location) of bowel in which it is expressed.

Expression Scores in strictures

The expression of TNFa was moderate in strictures of all patients. We did not observe any correlation of ES on the clinical outcome. TNFa is known to be present in significant measure in inflamed intestinal tissue as well as strictures. However, this was not observed in our cohort when compared to other cytokines we examined. It should be noted that all the patients in our cohort had been exposed to anti-TNF alpha therapy to treat their CD, within six months of the surgery and some until the time of surgery. Whether this had an influence on expression levels is worth considering.

Expression Scores in margins (proximal and distal segments)

In our cohort there was no observed correlation of ES in the margins with outcomes. Most patients had moderate expression with differing outcomes. FCD-020 had low expression in the proximal and high expression in the distal margin and had a poor outcome.

Expression Scores in strictures and margins

The collective ES across all segments did not have a correlation with clinical outcome. Only one patient had a high ES in the margin with poor outcome. It probably highlights the importance of microscopic inflammation in the margins. As mentioned before, whether exposure to anti-TNF α affects presence of TNF α in the tissue has to be kept in mind as a confounding factor. The drug very likely does have an inhibitory effect as the monoclonal antibody is designed to bind to the cytokine.

4.9 Potential implications of our findings from IHC

As part of this project, we have been able to successfully conduct IHC on a cohort of patients undergoing surgery for stricturing CD. This is a challenging cohort to recruit, and the availability of full thickness resections adds to the strengths of our methodology. We have carried out high quality IHC on multiple segments of the bowel wall from patients with a range of clinical outcomes. By analysing the images in detail using a novel scoring system, we correlated them to clinical outcomes. The implications of our findings are discussed in this section under 3 broad headings-

Potential Targets of therapy

IL-33

IL-33 has been considered a potential target of therapy for some years now. It has so far not been tested extensively in IBD. It has been tested in other autoimmune disorders such as atopic dermatitis. Results from clinical trials of monoclonal antibodies targeting IL-33 have been

unsuccessful. (Arena 2023) This reflects findings from several groups in preclinical models over the last few years reporting on the dual nature of IL-33 in immune regulation.

Our results show high expression of IL-33 in strictures and margins but no correlation with outcomes. It may well be involved in driving fibrosis, but it is challenging to infer this from our findings alone. Based on our current understanding, it would be difficult to recommend IL-33 as a target of therapy in IBD. Further work needs to be undertaken to understand its role better. It will always remain a challenge to harness a target which also has protective functions in the intestine, due to higher risk of treatment related adverse outcomes, seen with blocking IL-6 in CD. (Danese, Vermeire et al. 2019)

While targeting IL-33 shows promise in therapeutic applications, we recommend that caution is exercised to fully comprehend its complex functions and potential side effects. Continued research into IL-33's mechanisms and regulation will help us give a better understanding.

IL-36 gamma

IL-36g and its family have been potential targets of therapy in autoimmune disorders. A monoclonal antibody targeting the IL-36 receptor has been investigated in other autoimmune conditions. It has also been investigated in a clinical trial for patients with B3 phenotype of CD. The results are yet to be published.

Our results show high expression of IL-36g in strictures and margins of resection samples. High expression levels correlated with poor outcomes and moderate expression correlated with good outcome. The presence of IL-36g within the stricture makes it an attractive target of therapy. A monoclonal antibody against IL-36 is likely not only to have anti-inflammatory effect but also potentially anti-fibrotic effect, as proven in preclinical models of IBD. (Scheibe, Kersten et al.

2019) The high expression levels would also make it more likely that such patients would respond. Based on current understanding, IL-36g has proinflammatory effects in later stages which has to be borne in mind. It is perhaps better used in established diagnosis of IBD. It has been used successfully in pustular psoriasis (Bachelez, Choon et al. 2019) and would be interesting to see the results of the clinical trial in CD. It is likely to be a promising target in IBD.

As possible biomarkers to predict clinical outcomes post-surgery

IL-33

Our findings did not show any correlation with expression levels and clinical outcomes. There is not sufficient evidence to recommend using IL-33 alone as a biomarker to predict long term post-surgical clinical outcomes.

IL-36g

There was correlation of high expression of IL-36g in either strictures or resection margins, with disease recurrence. IL-36g when seen on IHC may act as a potential biomarker in predicting recurrence or adverse outcomes, but this requires further research.

TNF alpha

There was no correlation of expression levels of TNFa with clinical outcomes. Based on our finding, it is unlikely to have a role as a biomarker in predicting recurrence.

For patient stratification based on margins of resection

As part of this project, we wanted to study the effects of margins of resection on disease recurrence, as one of our secondary objectives. This is one of the surgical dilemmas we wanted to explore in more detail which is discussed in section 1.2.4. The presence of microscopic inflammation in resection margins in CD carries a higher risk of early post-operative endoscopic recurrence. (Poredska, Kunovsky et al. 2019)

On collective assessment of expression of selected cytokines in resection margins, the presence of microscopic inflammation (moderate or high expression of cytokines) at even one of the margins, was more likely to result in a poor outcome due to early disease recurrence. When IL-33 and/or IL-36g were detected in the margins, the outcome was poor. The role/presence of TNFa was not as clear for stratification.

Both IL-33 and IL-36g could be considered as biomarkers to stratify high risk patients after surgery. If resection margins show microscopic inflammation, such patients could be prioritised for more aggressive medical management during the post-operative monitoring period.

4.10 Strengths and limitations of the study

Our study has many strengths. This is a prospective observational study with a robust study design. There is reliable data on demographics, phenotype, medications, and patient journey. The data on clinical outcomes was updated during follow-up every 3-6 months and all adverse outcomes have been recorded at 6-monthly intervals for a minimum of 3 consecutive years. Despite it being a challenging cohort to recruit, a relatively large number were included from a single tertiary care centre. The access to tissue post-surgery gave us a rare opportunity to examine

full thickness resections, allowing us to make detailed observations of immunological changes occurring at the tissue level (deeper than mucosa which is a limitation of biopsies). Within the lab, we had expertise to carry out detailed work on transcriptomics and immunohistochemistry, using advanced technology and state-of-the-art equipment at the University. There was excellent bioinformatics support and we used well described, validated pipelines for analysis.

Our study also has some limitations. It was a relatively small cohort of patients that we finally selected for RNA analysis. This was done based on volume of tissue and other factors, probably introducing a degree of selection bias. However, we ensured that the lab team were blinded to the clinical details at all times. There were no external healthy controls in this study for full thickness resection tissue. This is a universal challenge as it would be ethically challenging to access normal healthy bowel surgically. To overcome this, we used resection margins as internal controls. The number of patients was also curtailed due to disruption of work by the pandemic. This has been discussed in detail at the start of the thesis. For the same reason, some of our genes of interest could not be confirmed using quantitative PCR, but this can be addressed in future work.

A smaller number of patients were selected for IHC work but the detailed work offsets this to some degree. Due to the limitations of numbers, it is challenging to claim wide and generalised applicability of our findings.

4.11 Conclusions

In our cohort of patients who underwent surgical resection for CD-related strictures, we have carried out detailed experiments using an 'omics' approach. This is a challenging cohort of patients to recruit, and it is also not very common to find many studies with detailed analysis of full-thickness bowel resection samples with this degree of depth. The linear scheme of this prospective study which ties robust demographics, clinical and follow up data with data on transcriptomics and immunohistochemistry makes it reliable and unique.

In our cohort, there is differential upregulation of genes specific to some JAK receptors and STAT family members within a CD-related stricture compared to non-strictured bowel. Our findings would suggest that in addition to being anti-inflammatory targets in IBD, they are likely to be potential anti-fibrotic targets in stricturing disease. This requires further work using validation by quantitative PCR. Further lab work using JAK1 selective inhibitors in ex-vivo models to examine their efficacy in inhibiting profibrotic signals will add to our understanding. Further testing of these drugs in animal models of stricturing disease are necessary before they are considered for use in clinical practice.

Our findings on IHC of novel target cytokines have given us interesting and mixed results. By applying a novel semi-quantitative scoring system for IHC and correlating them with clinical outcomes, our understanding of IL-33 and IL-36g have enhanced.

IL-33 is highly expressed in CD-related strictures and margins but does not influence outcomes. Because of its pleiotropic effects, it is unlikely to be a safe target of therapy in IBD, based on our findings.

IL-36g is highly expressed in CD-related strictures and margins, and this appears to result in poor outcomes. It is likely to be a safe target of therapy in later stages of stricturing CD.

Further ex-vivo work using monoclonal antibodies against both these cytokines is necessary to increase our understanding.

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6 Appendix 1

Table 6-1 Detailed analysis of correlation between different categories (individual layers)

		Correlation between		Significance/relationship	Pearson correlation	p-value
By	Individual layer	MUAI	MUCI	Y/Positive	0.339	0.019
		MUAI	SMAI	Y/Positive	0.519	0.000
		MUAI	SMCI	Y/Positive	0.492	0.000
		MUAI	MPCI	Y/Positive	0.312	0.031
		MUAI	SSCI	Y/Positive	0.318	0.029
		MUCI	SMCI	Y/Positive	0.432	0.002
		MUCI	MPCI	Y/Positive	0.438	0.002
		MUCI	SSCI	Y/Positive	0.294	0.045
		MUFI	MUMH	Y/Positive	0.618	0.000
		MUFI	SMFI	Y/Positive	0.429	0.002
		MUFI	SMMH	Y/Positive	0.525	0.000
		MUFI	SMNH	Y/Positive	0.381	0.007
		MUFI	MPMH	Y/Positive	0.360	0.012
		MUFI	MPVE	Y/Positive	0.353	0.014
		MUMH	MUVE	Y/Positive	0.537	0.000
		MUMH	SMCI	Y/Positive	0.296	0.041
		MUMH	SMFI	Y/Positive	0.407	0.004
		MUMH	SMMH	Y/Positive	0.598	0.000
		MUMH	SMNH	Y/Positive	0.451	0.001
		MUMH	MPMH	Y/Positive	0.413	0.04
		MUMH	MPVE	Y/Positive	0.389	0.006
		MUMH	SSCI	Y/Positive	0.350	0.016
		MUVE	SMMH	Y/Positive	0.327	0.023
		MUVE	MPFI	Y/Positive	0.350	0.015

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MUVE	MPMH	Y/Positive	0.479	0.001
SMAI	SMCI	Y/Positive	0.346	0.016
SMAI	MPAI	Y/Positive	0.576	0.000
SMAI	MPCI	Y/Positive	0.433	0.002
SMCI	SMVE	Y/Positive	0.338	0.019
SMCI	MPCI	Y/Positive	0.601	0.000
SMCI	MPMH	Y/Positive	0.418	0.003
SMCI	MPVE	Y/Positive	0.433	0.002
SMCI	SSCI	Y/Positive	0.628	0.000
SMCI	SSMH	Y/Positive	0.395	0.006
SMCI	SSVE	Y/Positive	0.295	0.044
SMFI	SMMH	Y/Positive	0.515	0.000
SMFI	SMAP	Y/Negative	-0.301	0.038
SMFI	SMNH	Y/Positive	0.310	0.032
SMFI	SMVE	Y/Positive	0.301	0.308
SMFI	SSFI	Y/Positive	0.411	0.004
SMMH	SMNH	Y/Positive	0.361	0.012
SMMH	SMVE	Y/Positive	0.359	0.012
SMMH	MPCI	Y/Positive	0.302	0.037
SMMH	MPMH	Y/Positive	0.486	0.000
SMMH	MPVE	Y/Positive	0.448	0.001
SMAP	MPCI	Y/Negative	-0.393	0.006
SMAP	SSCI	Y/Negative	-0.404	0.005
SMAP	SSFI	Y/Negative	-0.423	0.003
SMAP	SSNH	Y/Negative	-0.301	0.040
SMAP	SSVE	Y/Negative	-0.502	0.000
SMNH	MPNH	Y/Positive	0.349	0.015
SMNH	MPVE	Y/Positive	0.360	0.012
SMNH	SSNH	Y/Positive	0.354	0.015

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MPAI	SSAI	Y/Positive	0.406	0.005
MPCI	MPMH	Y/Positive	0.417	0.003
MPCI	MPVE	Y/Positive	0.343	0.017
MPCI	SSCI	Y/Positive	0.591	0.000
MPCI	SSFI	Y/Positive	0.337	0.020
MPCI	SSVE	Y/Positive	0.297	0.043
MPFI	MPMH	Y/Positive	0.311	0.031
MPMH	MPAP	Y/Positive	0.303	0.036
MPMH	MPVE	Y/Positive	0.677	0.000
MPMH	SSCI	Y/Positive	0.320	0.028
MPMH	SSFI	Y/Positive	0.398	0.006
MPMH	SSMH	Y/Positive	0.359	0.013
MPMH	SSVE	Y/Positive	0.293	0.046
MPNH	SSCI	Y/Positive	0.296	0.044
MPNH	SSFI	Y/Positive	0.295	0.044
MPNH	SSMH	Y/Positive	0.423	0.003
MPNH	SSNH	Y/Positive	0.561	0.000
MPNH	SSVE	Y/Positive	0.319	0.029
MPVE	SSCI	Y/Positive	0.403	0.005
MPVE	SSMH	Y/Positive	0.358	0.013
MPVE	SSNH	Y/Positive	0.289	0.049
MPVE	SSVE	Y/Positive	0.310	0.034
SSCI	SSFI	Y/Positive	0.489	0.000
SSCI	SSMH	Y/Positive	0.510	0.000
SSCI	SSNH	Y/Positive	0.406	0.005
SSCI	SSVE	Y/Positive	0.640	0.000
SSFI	SSMH	Y/Positive	0.380	0.008
SSFI	SSVE	Y/Positive	0.743	0.000
SSMH	SSNH	Y/Positive	0.462	0.001

SSMH	SSVE	Y/Positive	0.444	0.002
SSNH	SSVE	Y/Positive	0.412	0.004

MUAI, mucosa active inflammation; MUFS, mucosa fibrosis; SMFS, submucosa fibrosis; SMMH, submucosa muscular hyperplasia; SMNH, submucosa neuronal hypertrophy; MPMH, muscularis propria muscular hypertrophy; MPNH, muscularis propria neuronal hypertrophy; SSFI, subserosa fibrosis; SSVE, subserosa volume expansion

AI, active inflammation (global); CI, chronic inflammation; FS, fibrosis; MPH, muscular proliferation and hypertrophy

Y=yes

7 Appendix 2

7.1 Study protocol for TRAFIC Study

Study team at Birmingham

Professor Marietta Iacucci – Chief Investigator

Professor Subrata Ghosh – Previous Chief Investigator

Professor Thomas Pinkney – Senior Co-investigator

Dr. Uday Nagesh Shivaji – Current Principal Investigator

Dr. Samuel Smith – Co investigator and PhD PGR

Dr. Kassiani Skordilis- Consultant Pathologist

Dr. Davide Zardo – Consultant Pathologist

Dr. (Late) Louisa Jeffery – Postdoctoral scientist

Sarah Akiror (Dr) – PhD PGR

Professor Gergios Gkoutos- Professor in Bioinformatics

Dr. Animesh Acharjee- Senior Bioinformatician

Ms. Christine Bossa- Research Nurse

Ms. Rosemary Matonhodze- Research Nurse

Ms. Hollie Caulfield- Research coordinator

Ms. Suzanne Lockyer- Research coordinator

Ms. Gail Heritage- Research coordinator

Ms. Helen Duffy- Research coordinator

Study design

This will be a longitudinal, descriptive, non-interventional prospective cohort study to explore novel biomarkers associated with course of the disease

The patients in the three groups will be recruited at different time points in the journey of their disease. We hypothesise that the immune profiles of each group are likely to be different which

need to be studied in further detail. The possibility of describing a predictive biomarker would be greater when they are studied at distinct episodes of their condition e.g. new diagnosis, escalation of therapy, surgery. Each patient who is recruited into the study will be followed up for the duration of the study and the subsequent samples of blood or tissue will serve as internal comparators. This will help investigate the changes in immune profiles in the same patient as their disease progresses.

This is an exploratory study in an area that is currently lacking evidence. It is therefore difficult to power this study. The results from this study are likely to drive hypotheses of future studies and provide a basis to power studies or projects in the future.

For this exploratory study, we aim to recruit 180 patients in two years. Patients will be recruited from the Gastroenterology departments at University Hospitals Birmingham NHS Foundation Trust. The study will also have sites in India, which will conduct the study independently from the UK, but will contribute to the global recruitment and provide data

Safety reporting

No Treatment or intervention, other than bowel resection surgery as standard of practice, will be performed. Bloods are taken as part of routine clinical visits. Therefore, only clinical events will be recorded.

Ethical and regulatory considerations

Within the UK, the study will be conducted in accordance with the principles of Good Clinical Practice (GCP) that have their origin in the Declaration of Helsinki and are consistent with the UK Policy Framework for Health and Social Care Research. These principles protect and promote the interests of patients, service users and the public in health and social care research, by describing ethical conduct and proportionate, assurance-based management of health and social care

research, so as to support and facilitate high-quality research in the UK that has the confidence of patients, service users and the public.

All investigators within the UK and study site staff will comply with the requirements of the General Data Protection Regulation (GDPR) and Data Protection Act 2018 with regards to the collection, storage, processing and disclosure of personal information and will uphold the Act's core principles.

The CI will send Annual Progress Reports to the Main REC and the Sponsor, and report any serious breaches of the study protocol/research governance within 7 days. Any substantial amendments to the research will be submitted to the Main REC and the Sponsor before implementation and copied to the R&D, except in the case of urgent safety measures, which may be taken without prior approval.

Regulatory Review & Compliance

Within the UK, the study will be conducted in accordance with the UK Policy Framework for Health and Social Care, the applicable UK Statutory Instruments, (which include the Data Protection Act 2018 and Human Tissue Act 2008") and GCP. The protocol will be submitted to and approved by the main REC prior to circulation.

Before any patients are enrolled into the study, the Principal Investigator is required to obtain written confirmation of local R&D approval. Sites will not be permitted to enrol patients until confirmation of R&D approval is received by the TRAFIC Study Office and written confirmation from the Study Office confirms that recruitment may commence.

It is the responsibility of the Principal Investigator to ensure that all subsequent amendments gain the necessary local approval. This does not affect the individual clinicians' responsibility to take immediate action if thought necessary to protect the health and interest of individual patients.

Participating sites in India will conduct the study in line their guidelines and will obtain the necessary ethical approvals.

Insurance and Indemnity

The University of Birmingham will act as a single sponsor for UK sites only. As sponsor, the University is responsible for the general conduct of the study in the UK and shall indemnify the sites against any claims in the UK arising from any negligent act or omission by the University in fulfilling the sponsor role in respect of the study. The university is under no obligation to indemnify the sites against any claims arising from the conduct of the study centre.

In terms of liability, NHS Hospitals in the UK have a duty of care to patients treated, whether or not the patient is taking part in a clinical trial. Compensation will only be available in the event of clinical negligence being proven. There are no specific arrangements for compensation made in respect of any SAEs occurring through participation in the trial, whether from side effects listed, or others yet unforeseen.

Participating sites in India will be covered by their own sponsor and indemnity arrangements.

7.2 Study related documents approved by Research Ethics Committee

This section of the appendix contains study related documents that I designed for the study. I wrote the Patient Information Sheet (PIS) and the Informed Consent Form (ICF). The original versions of both documents are below.

7.2.1 Patient Information Sheets (PIS)

Patient Information Sheet for patients undergoing surgery



Tissue remodelling and fibrostenosis in Crohn's disease- a study of molecular signals and biomarkers

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and discuss it with others if you wish. Do ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Inflammatory bowel disease (ulcerative colitis and Crohn's disease) is a chronic condition which can have periods of remission and relapse characterised by inflammation of the bowel. Despite successful use of drugs in the treatment of these conditions, a significant number of patients still develop complications. Patients with Crohn's disease can develop complications e.g. - thickening and narrowing of bowel, intestinal obstruction. It is unclear as to what causes these complications in certain groups of patients. It is also unclear as to why some patients show excellent response to biological therapies and others do not. At present, there is no reliable way to identify high risk of complications before they occur or predict response to treatment.

The purpose of the study is to look into the biomarkers & chemical signals of inflammation and reasons for thickening & narrowing in surgically resected bowel, samples of intestinal biopsies and blood of patients with inflammatory bowel disease. The detailed analysis of such chemical signals are likely to provide new information and targets to develop newer therapies in the future.

Who is taking part?

Patients with inflammatory bowel disease at selected hospitals in the UK are taking part in the study

Why have I been chosen?

You have been approached because you will be undergoing surgery for inflammatory bowel disease (ulcerative colitis or Crohn's disease) as advised by your treating team.

Do I have to take part?

The decision to take part is entirely voluntary based on the information you have been given. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the treatment you receive in any way.

What happens if I decide to take part in this study?

We would ask that you read through this patient information sheet; if there is anything you do not understand please discuss this with a member of the research team. If you decide to take part in the study, we will ask you to sign a consent form. Once you have signed the consent form we will ask for your permission to collect the relevant samples from you.

Blood samples for research purposes will be taken at the same time as your routine care blood samples during clinic visits. The samples for research are in addition to standard care bloods and the total volume of blood at one sitting will not exceed 50mls (10 teaspoons).

Tissue samples for research will be taken at the same time as your routine surgery. The bowel tissue required for research is taken only after the affected part of the bowel has been removed and after the pathologists have taken the relevant samples required for your routine care. We would only use waste tissue samples (that would normally be discarded) for research purposes. The amount of tissue taken for research is as deemed appropriate by the pathologist.

Once we have collected the research samples they will be transported to the University of Birmingham laboratory. Your samples will be labelled with a study ID number so that you cannot be identified. Samples collected will help us to better understand the mechanism of Inflammatory Bowel Disease.

Ribonucleic acid (RNA) is the version of the deoxyribonucleic acid (DNA) code that your body uses to make proteins which control how your cells function. Differences in DNA and RNA may therefore help to explain why some people develop certain conditions and others do not. If you

agree to take part in this study, some of the tissue sample we collect may be used to determine the RNA code of the samples, however, this will not include determining the sequence of all or part of your DNA code.

Will I benefit if I take part?

This study is exploring new information. The results of the study will not directly benefit you. It is expected that the results will be useful to patients in the future. We are also not able to give you any information from the lab tests carried out on your samples donated to us.

Are there any risks or burdens involved in taking part?

There are no additional risks to you if you decide to take part in the study. As blood tests are generally part of your routine care, the study does not add any risks. The bloods we collect for research purposes will be taken at the same time as your routine bloods.

Will my taking part be kept confidential?

Any information about you will be collected and stored in adherence with the Data Protection Act 2018 and General Data Protection Regulation (GDPR). With your consent your information will be accessed by authorised representatives of research team. Your data will be given a code number so that you are not identified, please see below for details about how your information will be used.

A copy of your consent form will be sent to the Study Office at the University of Birmingham. This is to help check that the study is being conducted properly. The consent form includes your name, but no contact details, and will be stored in a locked cabinet separately from all other study data.

How will you use information about me?

We will need to use information from you and your medical records for this research project.

This information will include your:

- Date of birth
- Gender
- Details of your disease history and medication

People will use this information to do the research.

Your full patient records will only be available to your care team at your hospital. However, your medical records and other data collected as part of the study may be looked at by the research team, by the hospital Research and Development department, by the Sponsor (University of Birmingham), by regulatory authorities. This is to ensure that the study is being conducted properly, and for safety purposes where necessary.

People who do not need to know who you are will not be able to see your name or contact details.

Your data and samples will have a code number instead.

We will keep all information about you safe and secure.

Once we have finished the study, we will keep some of the data so we can check the results. We will write our reports in a way that no-one can work out that you took part in the study.

What are my choices about how my information is used?

You can stop being part of the study at any time, without giving a reason, but we will keep information about you that we already have.

We need to manage your records in specific ways for the research to be reliable. This means that we won't be able to let you see or change the data we hold about you.

Where can I find more about how my information is used?

- at www.hra.nhs.uk/information-about-patients
- ask a member of the study team for a leaflet

The University of Birmingham is UK Sponsor for the study, and the contact for any queries or concerns regarding the use of your information is:

The Data Protection Officer

Email: dataprotection@contacts.bham.ac.uk | Telephone: +44(0)121 414 3916

What happens at the end of the study?

Throughout the study, and when it ends, your hospital doctor and general practitioner will continue to treat your bowel problems

What happens to the results of the study?

We aim to publish the results of the study in medical journals and to present results at conferences. Your identity will not be revealed in any publications. The results may also be used in statistical tests, diagnostic tests and in the development of new treatments to help develop care for patients with IBD. If you would like to receive information on results please use the contact details at the end of this Patient Information Sheet.

Who is organizing and funding this research?

This study is funded by the National Institute of Health Research (NIHR) Birmingham Biomedical Research Centre, University of Birmingham and University Hospitals Birmingham NHS Foundation Trust, UK. The study is organized by the research team in the Institute of Immunology and Immunotherapy at the University of Birmingham.

What happens if I change my mind during this clinical study?

Taking part in this study is entirely voluntary and you can withdraw at any time without giving a reason. This will not affect the treatment that you may subsequently receive. You are entitled to request remaining laboratory samples to be destroyed and no further laboratory analysis to be performed. After your last visit no new data will be added.

Any results that have already been obtained from the samples during lab experiments are the property of the University of Birmingham and will be used anonymously to report our findings.

Who do I contact if I have more queries or need more information?

Your clinical care or research team will happily answer your questions on any occasion when you visit. If questions arise between visits you will be able to contact the center and either speak to somebody at that time or arrange to do so later at a mutually agreed time.

If you need to discuss anything related to the study, please contact

LOCAL RESEARCH TEAM CONTACT DETAILS

If you would like to discuss this research with health care professionals not involved in the project, you could discuss this with your hospital doctor or members of the Patient Advisory Liaison Support (PALS) team

LOCAL PALS DETAILS

Thank you for taking time to read this information sheet and considering entry into our study.

7.2.2 Informed Consent Form (ICF)

This is the original version of the informed consent form that I designed for the study.

PATIENT CONSENT FORM

Study Title: TRAFIC- Tissue remodelling and fibrostenosis in Crohn's disease – a study of molecular signals and biomarkers

Lead Investigator

Patient Initials

Study number

1. I confirm that I have read and understood the Patient Information Sheet (Version _____, Dated ____/____/____) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my future medical care and management being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the University of Birmingham NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to the collection, transfer and storage of my blood/tissue/saliva samples (as detailed in the Patient Information Sheet) for use in this research and future ethically approved research, including genetic studies. ☐

5. I understand that data collected during the study including my date of birth and initials, will be transferred to the University of Birmingham.

☐

Data collected on paper about me will be stored in a locked filing cabinet at a secure location at the University of Birmingham and electronic data will be stored in a database held on University of Birmingham servers. I agree to collection and storage of these data.

6. I agree to a copy of this consent form being sent to the study office at the University of Birmingham. I understand that the form will be stored in a locked cabinet separate from other study data.

7. I agree to take part in the TRAFIC study

☐

Name of patient:

Date: __ / __ / __ **Signature:**

Name of person taking consent:

Date:- __ / __ / __

Signature:

Please give a copy of the Participant Information Sheet and the completed Informed Consent Form to the Patient.

Please file the original Consent Form in your Investigator Site File and submit a copy to the Study Office at the University of Birmingham. A copy should be added to the patient notes.

7.2.3 Patient and public Engagement recruitment

This is the role description to recruit lay members and patients for study steering committee.

TRAFIC Project Steering Committee

Role Description for Lay Member

Title of study- Tissue remodelling and fibrostenosis in Crohn's disease – a study of molecular signals and biomarkers.

Post: Members of Steering Committee

Duration: July 2019 – December 2022

Meetings: Every 6 months approximately

Payment: Honoraria of £75 per half a day for attendance/work associated with the Committee will be offered. Reasonable travel expenses incurred for attendance will be reimbursed when claimed with receipts.

Background

Inflammatory bowel disease or commonly known as IBD used to cover conditions such as ulcerative colitis and Crohn's disease. IBD is a chronic condition which can have periods of remission and relapse characterised by inflammation of the bowel. Despite successful use of drugs in the treatment of these conditions, a significant number of patients still develop complications.

Crohn's disease is a condition which can lead to multiple complications in most patients e.g. intestinal obstruction, thickening and narrowing of the bowel. The ongoing cycle of inflammation, injury and healing can lead to formation of narrowed segments of bowel (process also known as tissue remodelling). It is unclear as to what causes these complications in certain groups of

patients. It is also unclear as to why some patients experience excellent response to biological therapies (infliximab, adalimumab, ustekinumab), whilst others do not. At present, there is no reliable way to identify high risk of complications before they occur or predict how well someone will respond to the treatment.

The purpose of this study is to look into the biomarkers (naturally occurring substance, molecule or gene used as an indicator of a biological process in the body) and chemical signals of inflammation to better understand the reasons behind thickening and narrowing of bowel. We plan to use surgically removed (resected) bowel, samples of intestinal biopsies and blood of patients with IBD, (after consent of patient to do so has been given) to look for these biomarkers. We hope this detailed analysis of results will provide new information and targets to develop better therapies in the future. The biomarkers will be compared among different groups of patients e.g. newly diagnosed patients, patients not yet exposed to biologics and patients exposed to different biologics.

Steering committee

Members of the steering committee are expected to take a broad view of certain specified topics and to take an active part in the committee meetings. The main duties will involve reading a small amount of material sent prior to each meeting and being prepared to discuss issues arising. We are particularly interested in your experiences of IBD and the management of your condition.

Main responsibilities of steering committee members-

1. To attend a committee meeting approximately once every 6 months which are usually held at the Institute of Translational Medicine and generally last approximately 1-2 hours.
2. To read material sent to you prior to each meeting. This may include minutes of previous meetings and short research reports.

3. At the steering committee meetings to contribute to discussion of the following:

- Patient information and consent
- Study design (for example, content of patient interviews, improvements that can be made)
- Comment on preliminary results
- Ideas about analysis, presentation and dissemination of results

The members of the steering committee will have access to the research team who can offer guidance on committee procedures and the review materials sent prior to meetings.

Person Specification

Factor	Essential	Desirable
Qualifications	None	None
Experience	None	Committee experience
Skills	Willing to familiarise yourself with medical and research language relevant to the field	To keep up to date with IBD related news via media and information provided by the research team.
Knowledge	Knowledge of perspectives based on your background (e.g. IBD patient, family member, parent or carer for someone with Crohn's Disease)	To have some understanding of research
Personal qualities	Good communicator	Self confidence in mixed group Willing to give constructive feedback to the team

Ability to listen to others and express
own views in a concise and focussed
way

Openness to other people's views

Please apply via post OR via email by sending a CV and a separate document with personal statement by **16th of June 2019**

Postal address

Dr. Uday Shivaji

Office 2, Ground floor

Institute of Translational Medicine

Birmingham, B15 2TH

OR

Via email to

██████████ (Helen Duffy) or

██████████ (Uday Shivaji)

Members of the committee will be selected on merits.

8 Appendix 3

8.1 Studies disrupted due to COVID-19 pandemic

This appendix contains background information and available data from the studies that were disrupted due to the COVID-19 pandemic.

There were two projects which we had initiated with an aim to do exploratory work. One of them was to examine the role of the Guanylate Cyclase (GC-C) pathway in IBD. During my discussions with the team which first reported this in IBD, I saw its potential as a biomarker as well as a target of therapy. We made good progress with this pathway and presented our initial results at conferences.

The second project was on drug repurposing in IBD. The aim was to test the potential of already approved and marketed drugs to be repurposed in IBD. Our initial results were promising and were presented. Unfortunately, the work was incomplete and remaining planned work had to be abandoned due to the negative impact of the pandemic on lab work and collaborative work. The background, methods and results are discussed in this section.

Parts of this chapter have been presented at the ECCO, DDW and UEGW conferences.

Jeffery L, Shivaji U, Zardo D, et al.

Sa1123 Regulators of the guanylate cyclase pathway are potential novel markers of mucosal healing in IBD (Jeffery, Shivaji et al. 2020)

doi- 10.1016/S0016-5085(20)31397-4

Shivaji U N, Jeffery L, Batis N, Iacucci M, Ghosh S.

P096 The anti-inflammatory effects of niclosamide on cytokines produced by PBMCs derived from IBD patients. (Shivaji, Jeffery et al. 2019)

Journal of Crohn's and Colitis. 2019;13(Supplement_1):S134-S.

8.1.1 Novel biomarkers of disease activity, mucosal healing and potential targets of therapy in IBD

8.1.1.1 Guanylate cyclase pathway and its role in IBD

8.1.1.2 Background and aim

Inflammatory bowel disease (IBD) is a chronic inflammatory condition which is increasing in incidence globally, including in newly industrialised nations and estimated to increase in incidence over the next decade.(Molodecky, Soon et al. 2012, Kaplan 2015, Ng, Shi et al. 2018) The management of patients with IBD and its resulting morbidity places a huge economic burden on the health systems.(Burisch, Jess et al. 2013) Research over the years has led to better understanding of the immunological pathways involved, which in turn has resulted in development of currently available therapies to treat the disease and many more are in development.(Neurath 2017) Current therapies approved for use in patients who fail conventional therapy are mainly in the form monoclonal antibodies.(Hanauer, Feagan et al. 2002, Rutgeerts, Sandborn et al. 2005, Sandborn, van Assche et al. 2012, Feagan, Rutgeerts et al. 2013, Sands, Sandborn et al. 2019)

Despite advances in therapies to treat both ulcerative colitis (UC) and Crohn's disease (CD) over the last two decades, a significant number of patients fail to respond to these drugs or subsequently lose response at some point during their disease course. The high failure rate presents a challenge to clinicians with only a limited number of drugs in the armamentarium against this condition at present. It is therefore imperative that research into finding newer targets of therapies continues to ensure novel treatment options become available to be used in the future.

Intestinal Guanylate cyclase C (GC-C) present in epithelial cells has been shown to have a role in gut homeostasis. GC-C serves as a receptor for ligands that activate downstream pathways, which are essential to maintain the secretory function of the gut. This has been well described. The downstream effects of activation of GC-C are due to production of cyclic guanosine monophosphate (cGMP). GC-C is encoded by the gene *GUCY2C*, mutations in which are implicated in Familial Diarrhoea Syndrome. This was reported in 2012 by Fiskerstrand et al when they studied a large family in Norway who were affected by this condition.(Fiskerstrand, Arshad et al. 2012) They studied 32 family members who were affected with chronic diarrhoea and for the first time reported a mutation of *GUCY2C* as the cause of diarrhoea. In the same group of patients, it was noted that some family members had a wide-ranging spectrum of phenotype of disease. 10 of the family members had undergone a laparotomy for suspected bowel obstruction, with 8 of them having confirmed ileal inflammation. In some cases, patients needed more than one operation to resolve symptoms, including ileo-caecal resections. A total of 9 family members either had suspected or confirmed diagnosis of CD and one had non-specific enteritis diagnosed.(Fiskerstrand, Arshad et al. 2012)

In this family, the authors reported that all affected family members with chronic diarrhoea had an inheritance pattern with a dominant heterozygous missense mutation in an autosomal gene in *GUCY2C*.(Fiskerstrand, Arshad et al. 2012) They further analysed that the mutation resulted in a gain of function increasing ligand-mediated activation of GC-C, with subsequent intracellular accumulation of cGMP. Therefore, this mutation would result in production of comparatively high levels of cGMP in response to normal levels of endogenous ligands. The increased levels of intracellular cGMP levels result in decreased intestinal sodium and water absorption and increased chloride secretion, which causes chronic diarrhoea.

It has been previously reported that GC-C signalling plays a role in maintaining intestinal barrier function (Han, Mann et al. 2011) as well as regulating inflammatory response. (Steinbrecher, Harmel-Laws et al. 2011)

In one comprehensive study, Steinbrecher and colleagues showed in a murine model of colitis that mice deficient in GC-C or guanylin were resistant to chemically induced colonic inflammation and also demonstrated that epithelial GC-C signalling had a role in regulating mucosal immune homeostasis in the intestine. (Steinbrecher, Harmel-Laws et al. 2011) These studies indicate the role of GC-C pathway in intestinal inflammation. However, it is not very clear yet as to what its role is at specific stages of IBD such as inflamed state (active disease), mucosal healing (remission/quiescent disease) or intestinal fibrosis.

There have been studies which have looked into the effects of guanylate cyclase and its link to fibrosis in certain disease conditions. Epithelial *GUCY2C* has been shown to have a role in regulating transforming growth factor- β (TGF β) secretion and activation of fibroblasts through Akt, in both murine and *in vitro* human fibroblast models. (Gibbons, Lin et al. 2013)

Soluble guanylate cyclase (sGC) has been shown to interfere with TGF β signalling, which is a well described and a key pathway of fibrosis. sGC activity increases cyclic guanosine monophosphate (cGMP) levels, inhibits SMAD-independent TGF β signalling and reduces fibroblast activation and collagen release. (Beyer, Zenzmaier et al. 2015) It has also been noted that GC-C mutation is a risk factor for Crohn's disease. *GUCY2C* and its activator, *GUCA2A* have been shown to be downregulated in IBD. *GUCY2C* signalling also plays a role in the signalling pathway for colorectal cancer as demonstrated in *in vitro* models. (Gibbons, Lin et al. 2013) With its signalling pathways having such varied and important biological effects, we wanted to explore it further in the context of IBD.

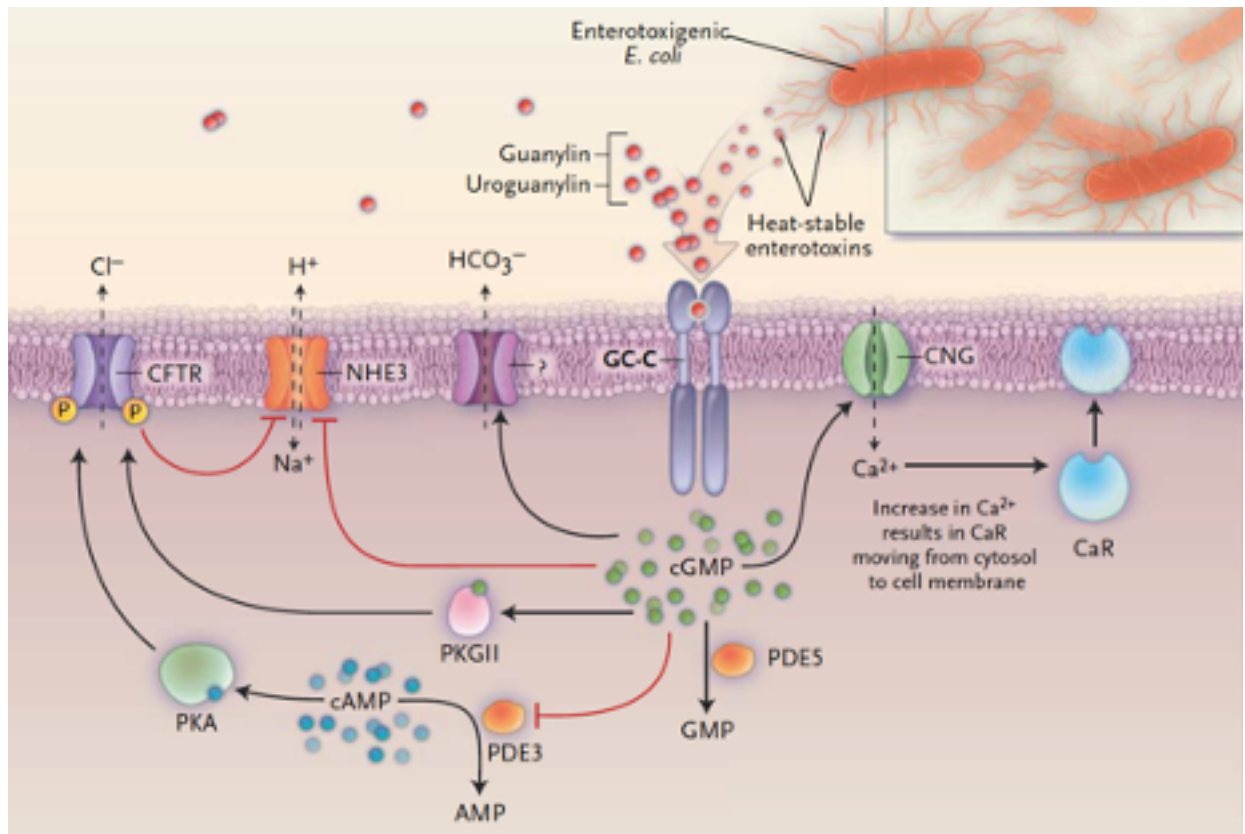


Figure 8-1 GCC pathway in the intestinal mucosa

8.1.1.3 Patients and Methods

Forty-four patients with IBD and 7 patients with polyps (controls) at University Hospitals Birmingham, UK were recruited under ethical consent. Relevant demographic and clinical data were extracted from the hospital EMR. All patients had disease activity recorded on endoscopic examination of mucosa and intestinal biopsies collected for analysis. Mucosal healing was defined as MES = 0 (UC) and SES-CD <6 (CD). Of 44 patients, 14 had matched baseline and 12-week post-biologic therapy assessment and had tissues collected. Intestinal biopsies were analysed by 3'RNA-sequencing using the Illumina Nextseq sequencer. FASTQ files were generated through BaseSpace and reads de-multiplexed, trimmed, aligned, and quantified using the GeneGlobe

(Qiagen) workflow. Expression was compared between groups using either Wilcoxon tests or Kruskal–Wallis with Dunn post-hoc analysis as appropriate.

8.1.1.4 Results

Expression of Guanylate cyclase activators GUCA2A and GUCA2B in patients who showed mucosal healing was equivalent to controls, but GUCA2A was downregulated in those with active disease (non-healing) ($p=0.006$). The same pattern was observed for transcriptional regulators of GUCY2C, including HNF4A ($p = 0.0248$) and CDX2 (0.0062).

Correspondingly, GUCY2C was reduced in non-healing mucosa, although the difference was not significant (Figure 8-2). In patients who responded to biologic therapy, both GUCA2A ($p = 0.0234$) and GUCA2B ($p = 0.0117$) were increased at follow-up but no change was observed for those who did not respond. Change in GUCY2C expression did not reach statistical significance in either group, although an increase was observed for a large proportion of responders.

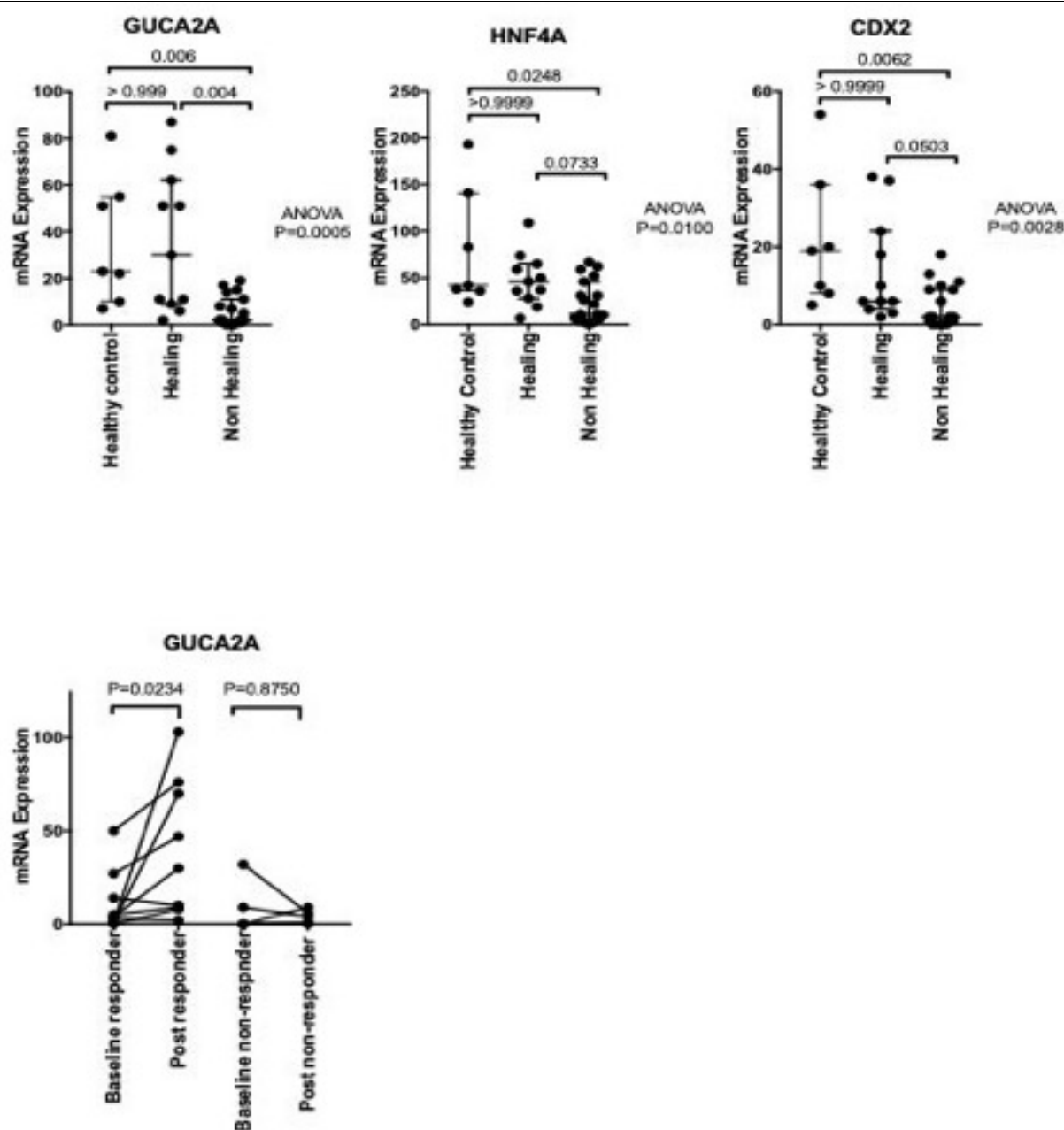


Figure 8-2 Results of GUCA2A, HNF4A and CDX2 expression in mucosal healing & response

8.1.1.5 Conclusions

Our findings suggest that regulation of the Guanylate Cyclase pathway may be involved in the restoration of a stable mucosa in IBD, and that expression of its regulators may be used to indicate response to treatment.

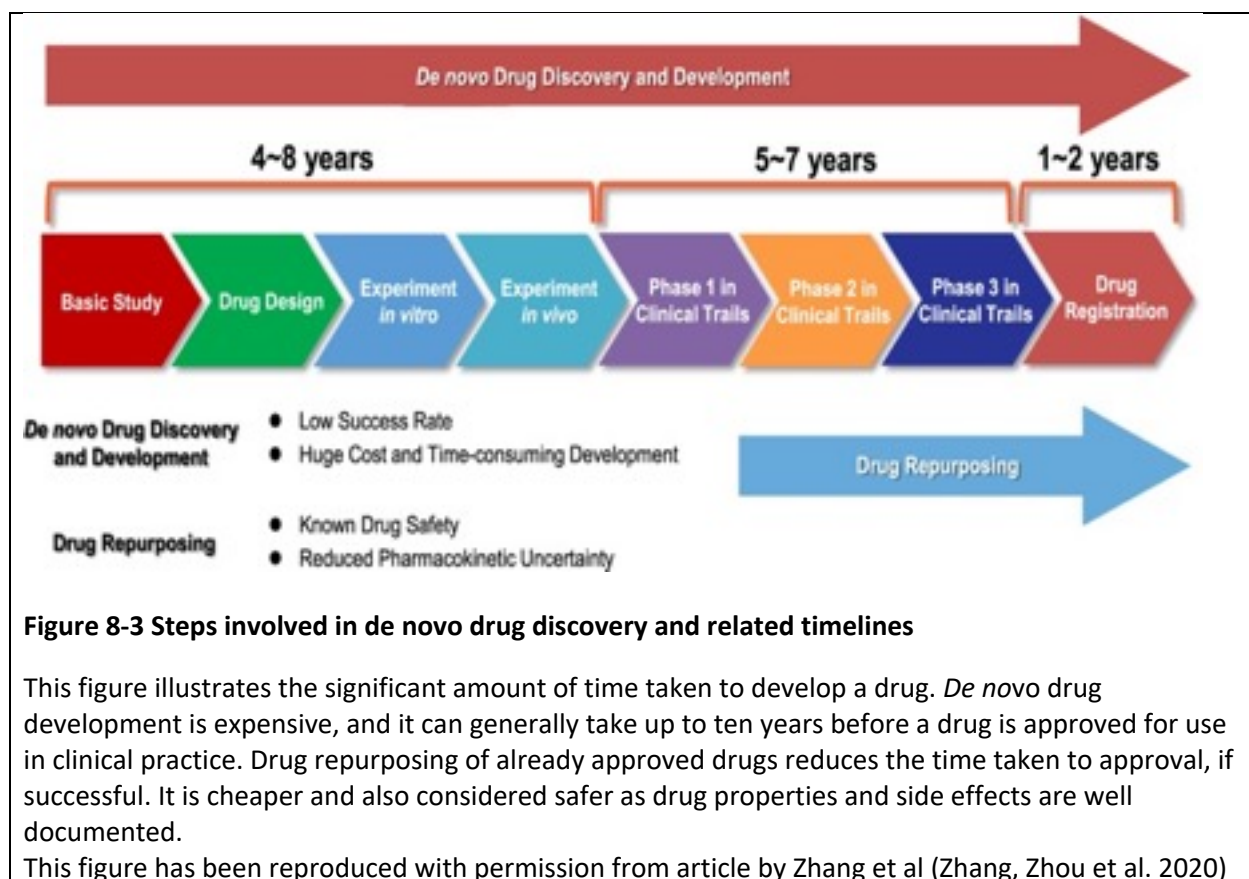
8.1.2 Drug repurposing in IBD

8.1.2.1 Background

Drug repurposing is a model that is now extensively used. As the development costs of new drugs and time to approval continue to increase, more and more drug companies are considering the possibilities of repurposing already used & approved drugs, mostly driven by economic feasibility.(Nosengo 2016) The principle of repurposing is based on the discovery of new indications for existing drugs, whether licensed or not, that are different to their original indications or therapeutic purposes.(HAM 2015) It is estimated that approximately 30% of drugs approved by the FDA in recent years have been repurposed drugs(Jin and Wong 2014) generating around 25% (\$500 billion) of the annual revenue of the pharmaceutical industry.(Naylor S 2015)

Advantages of drug repurposing

The main advantages of drug repurposing strategies are that the absorption, distribution, metabolism and excretion (ADME) pharmacokinetics, safety and toxicology of existing drugs are usually well characterised. Consequently, repurposed candidate drugs can often enter clinical trials more rapidly (estimated at ~6.5 years compared to 12-15 years), and at a considerably lower cost than newly developed drugs (estimated \$300 million compared to \$1.778 billion).(Duvvuri U 2009) Furthermore, since ~45% of drug candidates fail due to safety reasons, the known safety profile of many repurposed drugs increases the likelihood of a successful market launch. (Paul, Mytelka et al. 2010)



Niclosamide has recently been studied for its various other properties to potentially extend its use in other conditions. It has been seen to act as an immunomodulator and also thought to have anti-carcinogenic properties.

8.1.2.2 Niclosamide

Niclosamide is a salicylide which has been used as an anti-helminthic drug and appears to be minimally absorbed from the gastrointestinal tract. The accurate structure of this drug is described as a chlorinated salicylamide (2', 5-di-chloro-4-nitrosalicylamide). It has been used for over 60 years to treat most human tapeworm infections e.g.- *Taenia solium*, *T.saginata*, *Diphyllobothrium latum* and is approved for use by FDA and EMA. The current clinical preparations of niclosamide have been designed to be an effective anti-helminthic with a

resulting bioavailability of less than 10% following oral administration in humans. The oral dose of niclosamide for adult in cestocidal treatment is 2 g as a single dose, leading to peak serum concentrations of 0.25–6.0 µg/ml (~ 0.76–18.35 µM)(Andrews, Thyssen et al. 1982). The reason for this wide range of serum concentrations is considered to be due to intra-individual difference in absorption rate and poor water solubility, and hence diet plays a role as well.

Niclosamide has been studied and used in multiple conditions including cancer in recent years. It has been known to block or inhibit several pathways such as JAK/STAT, STAT3, Wnt and beta-catenin.(Osada, Chen et al. 2011, Li, You et al. 2013) Whilst most of these pathways have been studied in the context of cancer, some do overlap and there appears to be an interplay between cytokines which drive inflammation and fibrosis. It has also been shown to have an inhibitory effect on pro-inflammatory cytokines (TNF α , IL-6, IL-8, IL-17A, and IFN- γ), macrophages, dendritic cells and fibroblast-like synoviocytes in both *in vitro* and animal models.(Liang, Huang et al. 2015) The inhibitory effect it may have on TNF α pathways potentially make this a feasible option in selective patients.

8.1.2.3 Mechanism of action

Although it is not fully clear how the drug acts on the parasite, it is believed that niclosamide interferes with the energy metabolism of helminths possibly by inhibiting adenosine triphosphate (ATP) production, ultimately resulting in death of the parasite. This may be due to the inhibition of oxidative phosphorylation or by ATPase stimulation. There is considerable evidence of this but it may have multiple mechanisms of action. (Frayha, Smyth et al. 1997)

Niclosamide appears to be minimally absorbed from the gastrointestinal tract. At dosages used to treat helminthic infections, neither the drug nor its metabolites have been recovered from the blood or urine.(Gilman 1990)

Selective action

Human and helminth mitochondria are probably equally susceptible *in vitro* to niclosamide, but at therapeutic dose levels only parasite mitochondria are affected *in vivo*. This selectivity may also be due to the drug's poor absorbability, which restricts availability to host tissue. It is also observed that the drug inhibits glucose uptake which could be a direct effect or a result of reduced ATP production.(Frayha, Smyth et al. 1997)

Several studies have shown that niclosamide is active against cancer cells such as AML and colorectal cancer (Jin, Lu et al. 2010, Osada, Chen et al. 2011)and there is some evidence that niclosamide may have potential role on immune regulation. (Liang, Huang et al. 2015)

An experimental murine model study by Wu et al showed that niclosamide reduced the pro-inflammatory cytokine and chemokine expression of LPS-activated dendritic cells. It also affected the expression of MHC and costimulatory molecules and influenced the ability of the cells to take up antigens. In their model, niclosamide-treated dendritic cells showed decreased ability to stimulate T-cell proliferation and IFN- γ production. (Wu, Li et al. 2014) There was inhibition of LPS-induced DC maturation and cytokine. They also reported that blocking the LPS-induced activation of MAPK-ERK pathway, JN Kinase and NF-kappaB may contribute to the inhibitory effect of niclosamide on DC activation. They concluded that these results gave new insights into the immuno-pharmacological role of niclosamide and suggest that it may be useful for the treatment of chronic inflammatory disorders (Wu, Li et al. 2014)

A study which looked at in vitro use of niclosamide on synovial tissue of patients with rheumatoid arthritis showed that there was a significant decrease in TNF-alpha induced proinflammatory cytokines- IL-1 β , IL-6, IL-8, IL-17A and IFN γ in fibroblast like synoviocytes, in a dose-dependent manner.(Liang, Huang et al. 2015)

Our hypothesis is based on the immunomodulatory and anti-inflammatory properties noted in models of rheumatoid arthritis and cancer studies (drug used in phase 2 trials). It has been shown to have an effect on specific cell lines such as fibroblasts and dendritic cells which are recognised to play a significant role in inflammation and fibrosis in IBD. It is therefore a potential drug to control inflammation and reduce fibrosis & stricture formation.

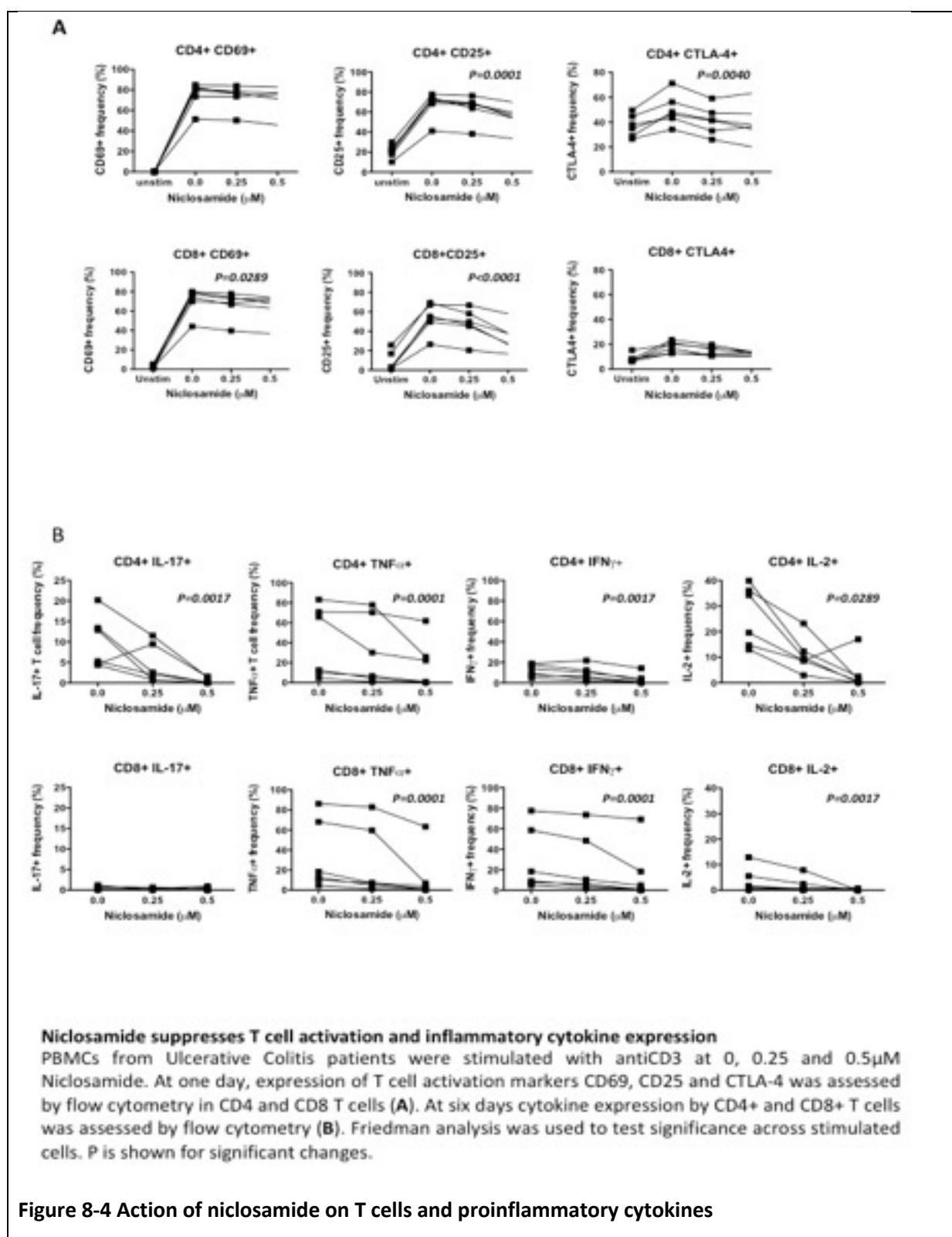
The aim of the study was to assess the effects of selected drugs e.g. niclosamide, on immune cells *ex vivo* and assess its potential for repurposing in IBD.

8.1.2.4 Patients and Methods

Peripheral blood mononuclear cells (PBMCs) from the bloods of 6 IBD patients were cultured with or without stimulation with 0.5 μ g/ml antiCD3 (clone OKT3). Niclosamide was prepared in dimethyl sulfoxide (DMSO) and diluted into culture medium at 0.25 μ M and 0.5 μ M. Effect of niclosamide upon cell survival and T cell activation was measured at 1 day by flow cytometry analysis of activation markers CD69, CD25 and CTLA-4. At six days, cells were re-stimulated with PMA and ionomycin in the presence of Brefeldin A and expression of cytokines IL-17A, TNF α , IFN γ and IL-2 measured by flow cytometry. Data was analysed by Flowjo and significance tested by Friedman Analysis.

8.1.2.5 Results

Niclosamide was not toxic to cells at the concentrations tested and did not alter the frequencies of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells or CD14⁺ monocytes in unstimulated cells. However, niclosamide reduced CD4 and CD8 T cell activation indicated by a significant decrease in the frequency of CD4⁺ cells expressing CTLA-4 and CD25 and CD69 and CD25 by CD8⁺ cells (Figure 1A). This resulted in a significant decrease in T cell number at six days ($P=0.0120$). Furthermore, it significantly inhibited expression of pro-inflammatory cytokines IL-17, IFN γ , TNF α and IL-2 by CD4 and CD8⁺ T cells. (Figure 1B)



8.1.2.6 Conclusion

Our results suggest a strong anti-inflammatory action of niclosamide when tested on T cells from IBD patients with no significant cell toxicity seen at concentrations used. The significant reduction in cytokine levels known to be involved in IBD make it a potential drug that could be used in IBD. Future work will focus on reproducing the results using intestinal tissue and also looking for effects as a potential anti-fibrotic agent.

