Exploring the Mechanisms of Fibrosis in Stenotic Phenotypes of Crohn's Disease

Dr Thomas Charles Shepherd MBBS MRCP

Institute for Liver and Digestive Health

Division of Medicine

University College London

Submitted for the degree of Master of Philosophy, July 2023

Resubmitted with corrections, February 2025

Supervisors:
Professor Massimo Pinzani
Dr Charles Murray
Professor Krista Rombouts

DECLARATION: I, Thomas Charles Shepherd, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

DEDICATION

This Thesis is dedicated to my father, Dr Hugh Arkwright Shepherd, who remains a constant source of encouragement and inspiration

"…Let him who is proud of his acumen and experience as a physician survey, from year to year, his own record in this respect, and his pride may take, will take, a severe fall…"

Dr Burrill Bernard Crohn 13th June 1884 – 29th July 1983

Exploring the Mechanisms of Fibrosis in Stenotic Phenotypes of Crohn's Disease

Dr Thomas Charles Shepherd MBBS MRCP

ABSTRACT

Background:

Up to 50% of patients with Crohn's disease (CD) will develop intestinal strictures involving and evolving from a process of currently unpreventable and irreversible fibrosis.

The pathogenesis of this selective fibrogenesis is unknown but involves complex pathophysiology.

Once initiated, characteristic TGF-β-driven myofibroblast (MF) activation and excess extracellular matrix (ECM) deposition is self-perpetuating.

Methods:

Proliferation, activation, and migration, with and without exogenous TGF-β stimulation, was investigated in primary human MF isolated from strictured and adjacent non-strictured ileum. Limited experiments on MF isolated from a

second CD patient with inflamed colonic disease were also performed synchronously until the viability of this culture was lost.

Culture of MF subtypes in isolation and in medium previously conditioned by prior incubation of another MF subtype (cross culture) was performed to assess paracrine effects.

RNA analysis of genes known to exert fibrotic influence was performed on biopsies of one patient taken from strictured and adjacent non-strictured intestine.

Results:

Proliferation

MF isolated from strictured ileum show a higher proliferative attitude.

Activation

However, MF activation, assessed by α-SMA expression, and collagen 1A expression, a significant ECM component, were greatest in MF isolated from non-strictured ileum compared to that of strictured ileum and colonic samples.

Cross Culture and conditioned media

Activation of MF isolated from strictured ileum was enhanced by culture in conditioned medium from incubated MF from non strictured ileum.

TGF-β treatment also enhanced activation and COL1A expression.

RNA gene expression

Profibrotic gene expression of ACTA2, COL1A, COL3A and TIMP-1 was increased in strictured intestine.

Non-strictured intestine had pronounced expression of other profibrotic genes, TGF-β, TNF-α, IL-6 and CCL2.

Summary:

These novel foundation experiments give new prominence to apparently macroscopically quiescent tissue adjacent to strictures. These areas could be considered biologically primed, and a reservoir of profibrotic mediators, involved in fibrosis initiation, development and progression.

IMPACT STATEMENT

The inflammatory bowel diseases (Crohn's disease, ulcerative colitis and IBD unclassified) affect more than 1.5 million in North America and 2.5 to 3 million populations in Europe, the latter at an estimated direct health care cost of 4.6-5.6 billion Euros per year.

In the United Kingdom, Inflammatory bowel disease (IBD) costs the NHS £720 million per year, in addition to the hidden cost of the majority of those affected being economically active.

Recent epidemiological studies have shown the incidence of IBD to be stable, and pertinent to this research, the prevalence of Crohn's disease (CD) has increased in the past 20 years from 220 to 400 per 100 000 persons.

Specific to this research, more than 30% of patients with CD will develop intestinal fibrosis resulting in stricture formation and subsequent intestinal dysfunction and obstruction associated with increased morbidity and mortality, causing prolonged hospitalisation and surgical intervention.

In contrast to the inflammatory component of Crohn's disease, where monoclonal antibody therapy has transformed medical treatment and thereby patient outcomes, a limited understanding of the mechanisms of fibrosis means

there is no current feasible therapy to prevent or reverse this process. This makes fibrotic CD a fertile area of research.

Experiments performed with the human myofibroblast presented in this thesis provide an opportunity for directly exploring a novel hypothesis of a component of fibrogenesis in CD.

The experimental concepts in this study offer not only a foundation for future research and publication but the possibility of extrapolation of the findings into other fibrogenic diseases, as yet unexplored.

Furthermore, mechanisms explored in this project, it is hoped can be considered a first step toward translational research and the potential of a new therapeutic target.

This is a piece of work that has involved my gaining a deep understanding of the current research arena and analysing the direction that this novel research should be taken.

The results, whether positive or negative, must be robustly valid, and it is anticipated will aid future research.

The ability to isolate and maintain viable myofibroblast subtypes in this laboratory would be fundamental to further progress.

Investigating the cell that is fundamental in the non-pathogenic or tissue healing pathway of fibrosis has the potential to provide the optimal opportunity of identifying any anomaly that might explain why over expression of this biological process progresses to a pathological one.

TABLE OF CONTENTS

PREFACE

This thesis represents original work by the author and has not been submitted in any form to any other University. Where use has been made of the work of others, this has been duly acknowledged in the text.

The research described in this thesis was undertaken at the Royal Free Campus, University College London (UCL), Institute for Liver and Digestive Health (ILDH).

Human myofibroblasts were donated by Professor Antonio Di Sabatino, First Department of Internal Medicine, St Matteo Hospital, University of Pavia, Pavia, Italy.

The work reported in this thesis was supervised by Professor Massimo Pinzani, Dr Charles Murray and Professor Krista Rombouts.

This independent work was funded by an Inflammatory Bowel Disease research bursary awarded from the Institute for Liver and Digestive Health, University College London.

ACKNOWLEDGEMENTS

This period of research was only made possible from funding by an awarded inflammatory Bowel Disease bursary from The Institute for Liver and Digestive Health, University College London.

I would like to convey sincere thanks to the following individuals, without whom this thesis would not have been possible.

If patience is a virtue, then my supervisors must be most virtuous – Professor Massimo Pinzani and Dr Charles Murray supported a determined clinician's desire to undertake laboratory based scientific research, with the challenge of human cells, and ambition to contribute something towards the understanding of inflammatory bowel disease. They provided unwavering guidance throughout my time in the laboratory, through to wholeheartedly supporting my career as a clinical gastroenterologist.

Even as the intervention of a consultant post halted this thesis, their encouragement and belief in me did not. Both have been nothing short of inspirational.

My co-supervisor, Professor Krista Rombouts, made the practicalities of this research possible, obligingly teaching a laboratory novice the techniques required for the delicate task of culturing and experimenting with human myofibroblasts and was never disparaging of even the simplest of questions.

Without the enthusiastic collaboration and donation of human myofibroblasts from one of the world leaders in intestinal fibrosis, Professor Antonio Di Sabatino at the University of Pavia, Italy, this work could have never commenced.

I am sincerely grateful to the patients from both Italy and the United Kingdom who participated.

An enormous debt of gratitude is owed to the scientists whose willingness to educate was invaluable.

A human cell in plastic can barely be left for a moment without attention and were it not for the unconditional help of Dr Lisa Longato, Dr Katrin Boettcher and Dr Pinelopi Manousou many more cells may have been lost in the name of science.

I particularly thank Lisa and Katrin for the numerous hours dedicated to teaching me western blot, and Giusi Marrone for the countless time spent guiding me through RNA isolation and analysis. And to Dr Giuseppe Mazza for his constant counsel.

FIGURES AND LEGENDS

Tables:

Images:

Figures:

ABBREVIATIONS

ACCENT 1	**A C**rohn's Disease **C**linical Trial **E**valuating Infliximab in **N**ew Long-Term **T**reatment Regimen
ACEi	**A**ngiotensin **c**onverting **e**nzyme **i**nhibitor
ACTA2	**Act**in **a**lpha **2**
BCA	**B**icin**c**honinic **a**cid
bFGF	**B**asic **f**ibroblast **g**rowth **f**actor
BrdU	**Br**omo**d**eoxy**u**ridine
BSA	**B**ovine **s**erum **a**lbumin
CCL2	Monocyte chemoattractant protein-1
CCL3	Macrophage chemoattractant protein-1
CD	**C**rohn's **d**isease
CDEIS	**C**rohn's **D**isease **E**ndoscopic **I**ndex of **S**everity
cDNA	**C**omplementary **d**eoxyribo**n**ucleic **a**cid
CM	**C**omplete **m**edium
cm	**C**enti**m**etres
CndM	**Co**n**d**itioned **m**edium
CO_2	Carbon dioxide
COL	**Col**lagen
COX	**C**yclo**ox**ygenase
CTE	**C**omputed **t**omography **e**nterography
CTGF	**C**onnective **t**issue **g**rowth **f**actor

DMEM	**D**ulbecco's **M**odified **E**agle **M**edium
DNA	**D**eoxyribo**n**ucleic **a**cid
dNTP	**D**eoxyribose **n**ucleoside **t**ri**p**hosphate
ECM	**E**xtra**c**ellular **m**atrix
EDTA	**E**thylene-**d**iamine-**t**etra-acetic **a**cid
ERK	**E**xtracellular signal-**r**egulated **k**inase
ELISA	**E**nzyme-**l**inked **i**mmuno**s**orbent **a**ssay
EMT	**E**pithelial to **m**esenchymal **t**ransition
Endo-MT	**Endo**thelial to **m**esenchymal **t**ransition
FBS	**F**oetal **b**ovine **s**erum
GAPDH	**G**lycer**a**ldehyde 3-**p**hosphate **d**e**h**ydrogenase
GIT	**G**astro**i**ntestinal **t**ract
H_2O	Water
HBSS	**H**ank's **b**alanced **s**alt **s**olution
hr	**H**ou**r**
IBD	**I**nflammatory **b**owel **d**isease
ICC	**I**ntestinal **c**ells of **C**ajal
IFN	**I**nter**f**ero**n**
IGF	**I**nsulin like **g**rowth **f**actor
IHC	**I**mmuno**h**isto**c**hemistry
IL	**I**nter**l**eukin
IUS	**I**ntestinal **U**ltra**s**ound

JAKi	**J**anus **k**inase **i**nhibitor
LOX	**L**ysyl **ox**idase
LPMCs	**L**amina **p**ropria **m**ononuclear **c**ell**s**
LPS	**L**ipo**p**oly**s**accharide
M	**M**olar
MAdCAM-1	**M**ucosal vascular **ad**dressin **c**ell **a**dhesion **m**olecule **1**
MDP	**M**uramyl **di****p**eptide
MF	**M**yo**f**ibroblast
mg	**M**illi**g**ram
ml	**M**illi**l**itre
mM	**M**illi**m**olar
MMP	**M**atrix **m**etallo**p**roteinases
MRE	**M**agnetic **r**esonance imaging **e**nterography
mRNA	**M**essenger **r**ibo**n**ucleic **a**cid
ng	**N**ano**g**ram
NHS	**N**ational **H**ealth **S**ervice
NICE	**N**ational **I**nstitute for Health and **C**are **E**xcellence
nm	**N**ano**m**etre
PAMPs	**P**athogen-**a**ssociated **m**olecular **p**atterns
PBS	**P**hosphate **b**uffered **s**aline
PCR	**P**olymerase **c**hain **r**eaction
PDGF	**P**latelet **d**erived **g**rowth **f**actor
PGE2	**P**rosta**g**landin **E2**

PVDF	**P**oly**v**inyli**d**ene **f**luoride
qPCR	**Q**uantitative **p**olymerase **c**hain **r**eaction
rcf	**R**elative **c**entrifugal **f**orce
RIPA	**R**adio**i**mmuno**p**recipitation **a**ssay buffer
RNA	**R**ibo**n**ucleic **a**cid
ROS	**R**eactive **o**xygen **s**pecies
rpm	**R**evolutions **p**er **m**inute
SD	**S**tandard **d**eviation
SDS-PAGE	**S**odium **d**odecyl-**s**ulfate **p**oly**a**crylamide **g**el **e**lectrophoresis
SEM	**S**tandard **e**rror of the **m**ean
SEMF	Intestinal **s**ub**e**pithelial **m**yo**f**ibroblast
SES-CD	**S**imple **E**ndoscopic **S**core for **C**rohn's **D**isease
SFM	**S**erum **f**ree **m**edium
SMA	**S**mooth **m**uscle **a**ctin
SMAD	**S**mall **M**others **A**gainst **D**ecapentaplegic
SMC	**S**mooth **m**uscle **c**ell
SNPs	**S**ingle **n**ucleotide **p**olymorphism**s**
STAR	**S**tenosis **T**herapy **A**nti-Fibrotic **R**esearch Consortium

TGF-β	**T**ransforming **g**rowth **f**actor beta
Th	**T**-**h**elper cell
TIMP	**T**issue **i**nhibitors of **m**etallo**p**roteinases
TLR	**T**oll-**l**ike **r**eceptor
TNBS	**T**ri**n**itro**b**enzene**s**ulfonic acid
TNF-α	**T**umour **n**ecrosis **f**actor alpha
TREAT	**T**herapy, **R**esource, **E**valuation, and **A**ssessment **T**ool
UK	**U**nited **K**ingdom
U/ml	**U**nits per **m**illi**l**itre
V	**V**olt
w/v	**W**eight per **v**olume
µg	Micro**g**ram
µm	Micro**m**etre

1: INTRODUCTION

1.1: Crohn's disease

Crohn's disease (CD), first described by Dr Burrill B. Crohn and colleagues in 1932, is an Inflammatory Bowel Disease (IBD), characterised by chronic relapsing and remitting immune mediated transmural gastrointestinal inflammation[1,2]. This inflammation can occur sporadically anywhere in the gastrointestinal tract (GIT), and complications include fistulae and strictures. Although primarily an intestinal disease, non-gastrointestinal systemic sequelae are observed, examples being pyoderma gangrenosum and enteropathic arthritis[3,4].

The aetiology is unknown, but multifactorial: Genetic predisposition, environmental factors, and the microbial flora of the gastrointestinal tract have all been shown to contribute to disease development, phenotype and progression[4].

1.1.1: Epidemiology

IBD affects more than 1.5 million in North America and 2.5 to 3 million in Europe[5], the latter at an estimated direct health care cost of 4.6-5.6 billion Euros per year[6]. In the United Kingdom, IBD costs the NHS £720 million per year (£3000

per patient per year), of which half relates to inpatient management[7]. In addition, there is significant morbidity and mortality in economically active people[7].

CD affects both men and women in equal proportion, with a median age of onset of 30 years[8,9].

For uncertain reasons, following a substantial rise in incidence in high-income western countries in the latter half of the 20th century, more recent epidemiological studies have shown the overall incidence of IBD to have stabilised, while rising rapidly in newly industrialised countries[10].

A stable incidence of CD in the United Kingdom of 14.3 (14.0-14.7) per 100 000 person-years, however, is contrasted by a rising prevalence, with an increase in the past 20 years from 220 to 400 per 100 000 persons[11].

The incidence of IBD remains constant, even less over the past decade, and this divergence between incidence and prevalence, has not been explained[12,13].

One explanation is that with better treatments and management, patients are indeed living longer. The capacity to treat inflammation will almost certainly have little or no effect on the cohort of CD patients that have the fibrotic or penetrating phenotype.

Research that is directed towards fibrosis may indeed become an important and complementary dimension of the research that is directed to IBD.

1.1.2: Natural history

The predominant phenotypic elements of CD are age of onset, disease location, and disease behaviour; and CD is typically classified by the Montreal revision of the Vienna classification (Table 1)[14].

Age at diagnosis	**A1**	Below 16 years
	A2	Between 17 to 40 years
	A3	Above 40 years
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**

Table 1. Montreal classification of Crohn's disease: Montreal classification of Crohn's disease: *L4 is a modifier that can be added to L1-L3 when concomitant upper gastrointestinal disease is present; **p is added to B1-B3 when concomitant perianal disease is present[14].

Symptomatic manifestations vary between individuals, and are often phenotype dependent, but commonly include abdominal pain, frequent diarrhoea, and weight loss[15].

Involvement of the terminal ileum alone or together with colonic disease, account for 50% of all cases[16].

The vast majority (80%) of individuals present with non-stricturing, non-penetrating or non-fistulising, inflammatory CD[17].

However, adversely, 11% of patients have disease already complicated by fibrotic strictures at initial presentation[18–20].

The risk of developing a stricture or fistulation complicating CD in any individual is substantial: 18% at 90 days; 22% at 1 year; 34% at 5 years; and 50% at 20 years[21,22].

In isolation, one population-based cohort study demonstrated the cumulative probability of progressing to stricturing disease to be 15% at 10 years and 22% at 20 years[23], although other studies reported higher rates of between 30 and 50% within 25 years[18,19].

Currently there are no accurate predictive factors for developing stricturing CD, although the prospective multicentre TREAT registry and the ACCENT 1 trial (see abbreviations) identified disease duration, severity of disease, ileal location and new corticosteroid use as risk factors[24,25].

Of importance, it is unlikely that the fibrotic stricturing is merely the consequence of a chronic wound healing reaction[26], as successful treatment and rapid resolution of active inflammatory CD with anti-tumour necrosis factor alpha (anti-TNFα) can be still associated with high incidence of transmural fibrostenotic disease[27,28]. A plausible explanation is that anti-TNFα treatment tends to resolve submucosal inflammation and the defective wound healing (by reducing the excess of matrix metalloproteinases[29,30]), whereas deeper, transmural inflammation is not affected by anti-TNFα[31–33].

Strictures are most common in the small bowel but are also seen in the colon where there is a higher association with dysplasia potentially progressing to malignancy[34].

1.1.3: Treatment

There is no cure for CD, and the objective of medical therapy is to interrupt the natural history and induce and maintain remission with fewer medical interventions and without the need for surgery[35].

Medical intervention ranges widely from periods of exclusive enteral (liquid) diet and brief courses of corticosteroids to long-term immunomodulation[36,37].

Treatment decisions are influenced by disease severity, location of disease and clinical phenotype combined with an attempt to predict those at risk of more aggressive progression[38].

Aggressive risk factors include, young age at diagnosis, extensive anatomical involvement, deep ulceration, previous surgical intervention and the additional complications of fistulae and stricture[34].

Meta-analysis has shown minimal benefit from the traditional inflammatory bowel disease medication, bowel specific aminosalicylates, in CD[39].

The foundation of medical therapy has become immunosuppression[40].

Options are limited to little more than a handful of agents:

a. Thiopurines (azathioprine and 6-mercaptopurine)[41];
b. Methotrexate[42];
c. Anti-TNF, primarily infliximab and adalimumab[43];
d. Adhesion molecule inhibition, vedolizumab (anti-$\alpha 4\beta 7$-heterodimer)[44,45];
e. Interleukin-23 (IL) inhibition, ustekinumab (anti-p-40 subunit of IL-12 and IL-23) or risankizumab (anti IL-23A)[46,47];
f. Janus kinase inhibitor (JAKi), upadacitimib[48].

Whilst the thiopurines proved to be a mainstay of immunosuppressive CD treatment since the Sixties, a Cochrane review (2016), has shown azathioprine and 6-mercaptopurine to offer no advantage over placebo for the induction of remission, and more recently supported by data attesting a lack of efficacy to alter the natural history of the disease[41].

Their contribution now limited to maintenance therapy in mild to moderate inflammatory phenotypic disease, or combination therapy with anti-TNF□, providing steroid free remission superior to anti-TNF□ monotherapy[41,49].

Of note, the contemporary use of therapeutic drug monitoring has finessed dosing and medication decisions[50,51].

A recent systematic review and meta-analysis also determined methotrexate to have little or no role for induction of remission but confirmed parenteral methotrexate to be efficacious in the maintenance of remission[42].

Anti-TNF□ therapy was approved for use in CD in 1998 challenging the standard choice for acute medical treatment[43].

Significantly improved rates of response were observed, and it remains the most effective treatment for moderate to severe inflammatory CD and penetrating CD: inducing remission in 50 to 70% and maintaining remission in more than 40%; and fistula closure reported in 50%[24,43,52].

Within the last decade vedolizumab (NICE approval 2015)[44], ustekinumab (NICE approval 2017)[46], risankizumab (NICE approval 2023) and upadacitinib (NICE approval 2023)[48] have all been licenced for use in treatment of CD and early real-world data suggests both are effective safe treatment options, although presently, in the United Kingdom at least, these agents are reserved for use in patients who have failed to respond to, or lost response to, or where there is a contraindication to TNF-□ inhibition, for example, active malignancy[43].

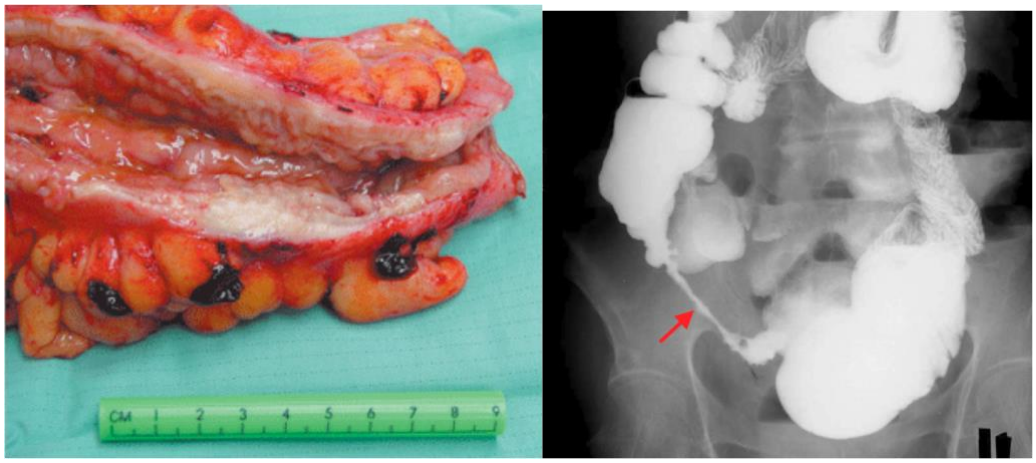
Most importantly, advances in medical therapy have not impacted on rates of surgical intervention, which can be substantially attributed to their lack of efficacy in treating or preventing stricturing disease, and which ultimately leads to stenosis and intestinal malfunction and obstruction[53,54].

Population-based cohort studies describe a cumulative risk of surgery between 40 and 71% within 10 years of diagnosis. Surgery is not curative for CD and many patients will require multiple surgical interventions over their lifetime[55–57].

1.2: Fibrostenosing phenotypes

Historically, stricturing CD disease was believed the more indolent sibling to penetrating disease, despite a paucity of evidence for this concept, it is a perspective reflected in the established Vienna or Montreal Classifications of CD, where patients with fistulae are scored as having the highest level of disease complication irrespective of the presence of a stricture, and only classified as having a stricture if this is the only complication present[21,58,59].
Therefore, the incidence of strictures using these classifications is likely to be underestimated[60].

Strictures, resulting in the clinical picture of intestinal malfunction with or without obstruction, occur as a complication of CD. Inflammation of the intestinal wall alone can result in luminal narrowing, but a proportion of patients' disease will be complicated by the development and progression of, as of yet an, irreversible transmural fibrosis resulting in stricture formation and stenosis[19,31,61].



A B

Image 1. Surgically resected fibrotic stricture from a patient with Crohn's disease (A). Contrast follow through demonstrating a stricture in a patient with Crohn's disease (B).

The fibrosing phenotype disproportionately influences the natural history of CD in these patients, manifesting with more pain and intestinal dysfunction, which correlates to earlier and multiple surgical interventions[60].

Comparable to distribution of inflammatory CD, the most common location for de novo strictures is in the ileum and adjacent colon (40 to 55%)[62,63], but can develop in any location affected by CD (25–40% ileum; 15–25% colon; 10% upper gastrointestinal tract)[60,64,65].

1.2.1: Stricture diagnosis

Symptoms correlate imperfectly with the presence of a stricture and when suspected, further investigation is essential[19].

Endoscopy

The endoscopic scoring systems SES-CD and CDEIS define stenosis as an area that is impossible or difficult to pass with an adult colonoscope[66], while the CrOhN's disease anti-fibrotic STRICTure therapies (CONSTRICT group), to standardise diagnosis, has proposed the endoscopic definition of a stricture as inability to pass an adult colonoscope through the narrowed area without prior endoscopic dilation with a reasonable amount of pressure applied[54,67].

Histology

Biopsies should be performed to aid in the exclusion of relatively superficial dysplasia, but of course, will miss the potential for neoplastic change in deeper layers of the diseased intestinal wall[68].

A large retrospective French study discovered dysplasia or malignancy in 2.4% of colonic stricture resection specimens, not previously identified by thorough endoscopic biopsy sampling[69].

Although there are currently no validated histological scoring systems to quantify severity of fibrosis[70], in 2023 the Stenosis Therapy Anti-Fibrotic Research (STAR) Consortium evaluated the appropriateness of histopathology scoring systems and published a summary of consensus key findings (Table 2)[70].

Gross morphology	Naïve and anastomotic strictures can be defined by gross examination alone Increased bowel wall thickness, decreased internal circumference and decreased luminal diameter must be present to diagnose a stricture Increased bowel wall thickness should be defined as measurement from the serosal aspect to the mucosal aspect Decreased internal circumference should be defined as an internal circumference measurement less than that of the uninvolved bowel wall Decreased luminal diameter should be defined as decreased luminal diameter measurement compared with the uninvolved bowel wall Creeping fat and fat wrapping should be defined as white adipose tissue extension from the mesenteric attachment to partly or wholly cover the antimesenteric aspect of the intestine
Histopathology	A naïve or anastomotic small bowel stricture cannot be defined on microscopic examination alone A minimum of one cross section should be taken per centimetre length of stricture H&E staining must be performed before making a diagnosis of small bowel stricture on pathology Fibrosis of the submucosa is required to diagnose a small bowel stricture and should be defined as an increased collagen content in the stricture section compared with any non-stricture section A small bowel stricture on microscopic examination may be accompanied by the following changes: • Increased thickness of wall layers (from serosa to mucosa) compared with adjacent healthy bowel wall • Fibrosis of the submucosa or the bowel wall • Muscularisation of the submucosa or increased thickness of the muscularis mucosa compared with adjacent healthy bowel wall Active mucosal inflammatory disease should be defined as: • Neutrophilic inflammation in the lamina propria or any epithelium • Erosion or ulcer • Fistula Chronic mucosal inflammatory disease should be defined as: • Crypt architectural distortion or crypt loss • Pyloric gland metaplasia • Paneth cell hyperplasia • Basal lymphoplasmacytosis, plasmacytosis or fibrosis • Prominent lymphoid aggregates at the mucosa/submucosa interface Transmural inflammation should be defined as inflammation across all four layers of the bowel wall, and includes lymphoid aggregates, granulomas and fistula tracts, but not neutrophilic inflammation No existing histopathological score for grading inflammation and fibrosis in small bowel stricturing Crohn's disease was considered appropriate

Table 2. Summary of appropriate key findings on histopathological scoring of small bowel stricturing disease[70]. *(Reproduced from Gut May 2021; I. Gordon et al)*

Radiology

Intestinal ultrasound (IUS), computed tomography enterography (CTE) and magnetic resonance imaging enterography (MRE) can identify stenosis with varying degrees of accuracy[71].

MRE appears most accurate in the evaluating the degree of fibrosis and inflammation within a stricture, differentiating mild-moderate and severe fibrosis with a sensitivity of 94% and specificity of 89%[31,66].

The CONSTRICT group define a stricture on cross sectional imaging as having and area characterised by localised luminal narrowing, bowel wall thickening and pre-stricture dilatation[54].

1.2.2: Defining a stricture

Despite clinical acumen and advances in investigative modalities and techniques, until recently there was no established consensus definition of a CD stricture and consequentially no consistent recommendations on diagnosis and management in clinical practice[72].

Broadly defined, a stricture is a section of bowel with wall thickening, luminal narrowing and prestenotic dilation, which requires cross-sectional imaging for

accurate diagnosis and to assess the degree of coexistent active inflammation[67,72].

The complexity of this clinical phenotype was highlighted in August 2024 by the by the STAR Consortium, who ultimately published 9 descriptive statements regarding the clinical approach for the diagnosis of fibrostenosing CD including a compilation of clinical symptoms, endoscopic findings, histopathology and dedicated bowel imaging (Table 3)[72].

.

Statement 1: Symptoms indicative of the presence of fibrostenosing Crohn's disease are cramping, dietary restrictions or changes, vomiting and abdominal pain after eating.
Statement 2: Symptoms are not required to diagnose fibrostenosing Crohn's disease.
Statement 3: In clinical practice, fibrostenosing Crohn's disease cannot be accurately diagnosed by clinical symptoms, physical examination, laboratory investigations, kidney, ureter, bladder plain radiography or endoscopic mucosal biopsies.
Statement 4: In clinical practice, fibrostenosing Crohn's disease can be accurately diagnosed by CT, MRI, IUS, endoscopy, intraoperative assessment by the surgeon and full-thickness histopathology.
Statement 5: CT, MRI, IUS or endoscopy are required for the diagnosis of fibrostenosing Crohn's disease.
Statement 6: Fibrostenosing Crohn's disease on CT, MRI or IUS is best defined by luminal narrowing, pre-stricture dilation and wall thickness.
Statement 7: The inability to pass an adult or paediatric colonoscope is required to diagnose fibrostenosing Crohn's disease.
Statement 8: Cross-sectional imaging using CT, MRI or IUS, as well as endoscopy and mucosal biopsies, may assist in identifying active inflammation in fibrostenosing Crohn's disease.
Statement 9: Currently, no cross-sectional imaging modality is able to accurately determine the degree of fibrosis in fibrostenosing Crohn's disease.

Table 3. Consensus statements on definitions and diagnosis of fibrostenosing Crohn's disease[72]*. (Reproduced from Nature Reviews August 2024; D. Bettenworth et al)*

1.2.3: Stricture treatment

No medical therapy reverses fibrotic stenosis and once an individual becomes symptomatic physical intervention is required[31,73].

An option for short (< 5cm) endoscopically accessible strictures, in the absence of concurrent fistulation or malignancy, is balloon dilatation[74]. This minimally invasive intervention has been shown to be effective at symptom relief in 80% of cases; however, the requirement for repeat dilatation is substantial (37 to 74%) with a diminishing success rate with each procedure[75].

This treatment modality provides only an interruption of this fibrosis complication, and ultimately 43% of patients will progress to surgical resection within 2 years[31].

Surgical options include segmental resection or stricturoplasty[76].

Stricturoplasty for short (< 10 cm) to medium (up to 25 cm) length strictures offers the benefit of being bowel-sparing, and, curiously, recurrence at the site of stricturoplasty is uncommon[77].

Larger strictures, or where stricturoplasty is not possible, may require segmental resection, but must this be tempered by a requirement to preserve

bowel length to prevent short bowel syndrome and potentially intestinal failure[78].

Furthermore, and in contrast to stricturoplasty, post-operative CD recurrence at the anastomosis commonly occurs, and a third of patients will require a second surgery by 10 years[79].

In the first post-operative year endoscopic (rather than symptomatic) recurrence is reported in 35 to 85% of cases and by the third year 85 to 100%[80].

Surgical intervention shortly after diagnosis is associated with longer clinical remission, reduced risk of repeat surgery and decreased exposure to corticosteroids and monoclonal antibody therapy and can be the first choice of management in younger patients presenting with terminal ileal CD[40].

In 2024, the STAR Consortium published the first consensus strategic statements for appropriate medical, endoscopic and surgical treatment (Tables 4, 5 and 6)[72].

Statement 1: Cross-sectional imaging is required prior to making any treatment decision in naive or anastomotic fibrostenosing Crohn's disease.
Statement 2: Patients with Crohn's disease with a naive or anastomotic fibrostenosing disease phenotype should undergo evaluation to assess for the presence of active inflammation prior to any intervention.
Statement 3: No drug with proven specific intestinal anti-fibrotic effect is available.
Statement 4: Patients with confirmed intestinal obstruction due to naive or anastomotic fibrostenosing Crohn's disease should be hospitalized and treated by a multidisciplinary team.
Statement 5: Anti-inflammatory medical therapy should only be considered if an active inflammatory component was confirmed in a patient with naive or anastomotic fibrostenosing Crohn's disease.
Statement 6: Bio-naive patients with symptomatic naive fibrostenosing Crohn's disease can be treated with corticosteroids, anti-TNF agents with or without immunomodulators, ustekinumab, endoscopic balloon dilation or surgery.
Statement 7: Bio-naive patients with symptomatic anastomotic fibrostenosing Crohn's disease can be treated with corticosteroids, anti-TNF agents with or without immunomodulators, ustekinumab with or without immunomodulators, endoscopic balloon dilation or surgery.
Statement 8: Bio-naive patients with asymptomatic naive fibrostenosing Crohn's disease can be treated with anti-TNF agents with or without immunomodulators or ustekinumab.
Statement 9: Bio-naive patients with asymptomatic anastomotic fibrostenosing Crohn's disease can be treated with anti-TNF agents with or without immunomodulators or ustekinumab.
Statement 10: In patients with anti-TNF treatment failure, symptomatic naive fibrostenosing Crohn's disease should be treated with ustekinumab, endoscopic balloon dilation or surgery.
Statement 11: In patients with anti-TNF treatment failure, symptomatic anastomotic fibrostenosing Crohn's disease should be treated with corticosteroids, ustekinumab with or without immunomodulator, endoscopic balloon dilation or surgery.
Statement 12: In patients with anti-TNF treatment failure, asymptomatic naive or anastomotic fibrostenosing Crohn's disease should be treated with ustekinumab or endoscopic balloon dilation.

Table 4. Consensus statements on medical treatment of fibrostenosing Crohn's disease[72]***. (Reproduced from Nature Reviews August 2024; D. Bettenworth et al)***

Statement 1: Cross-sectional imaging (CT, MRI or IUS) should be performed before any endoscopic intervention in a patient with naive or anastomotic fibrostenosing Crohn's disease.
Statement 2: A reasonable treatment approach for short (<5 cm) naive fibrostenosing Crohn's disease is endoscopic balloon dilation, strictureplasty and intestinal resection.
Statement 3: A reasonable treatment approach for short (<5 cm) anastomotic fibrostenosing Crohn's disease is endoscopic balloon dilation and intestinal resection.
Statement 4: A reasonable treatment approach for long (>5 cm) naive fibrostenosing Crohn's disease is strictureplasty and intestinal resection.
Statement 5: Endoscopic dilation therapy in naive or anastomotic fibrostenosing Crohn's disease is contraindicated in the presence of deep ulcers within the stricture, associated penetrating complications or malignant alterations associated with the stricture.
Statement 6: Naive or anastomotic fibrostenosing Crohn's disease longer than 5 cm should not be treated by endoscopic dilation therapy.
Statement 7: For patients with naive or anastomotic fibrostenosing Crohn's disease, endoscopic dilation using antegrade deployment of the through-the-scope balloon (not passing the stricture first, but pushing the deflated balloon through the stricture lumen in an antegrade fashion prior to inflation) is the preferred technical approach.
Statement 8: The recommended time of balloon insufflation during endoscopic dilation of naive or anastomotic fibrostenosing Crohn's disease is 60–90 seconds.
Statement 9: The most adequate maximum balloon diameter at the end of the endoscopic dilation therapy of naive or anastomotic fibrostenosing Crohn's disease is 15–18 millimetres.
Statement 10: The recommended maximum number of steps for graduated dilation during one sitting for naive or anastomotic fibrostenosing Crohn's disease is three.
Statement 11: After successful endoscopic dilation therapy of naive fibrostenosing Crohn's disease, the time to re-assessment should be determined by clinical symptoms, endoscopic appearance of the stricture and cross-sectional imaging appearance of the stricture.
Statement 12: After successful endoscopic dilation therapy of anastomotic fibrostenosing Crohn's disease, the time to re-assessment should be determined by clinical symptoms and cross-sectional imaging appearance of the stricture.
Statement 13: In patients with naive or anastomotic fibrostenosing Crohn's disease, medical anti-inflammatory therapy should be escalated after dilation if active inflammation is visible within the stricture at the time of dilation.
Statement 14: In appropriate patients with naive fibrostenosing Crohn's disease, endoscopic balloon dilation has a high technical success rate, a favourable short-term clinical efficacy and an acceptable complication rate.
Statement 15: In appropriate patients with anastomotic fibrostenosing Crohn's disease, endoscopic balloon dilation has a high technical success rate, a favourable long-term clinical efficacy and an acceptable complication rate.
Statement 16: Serial dilation of recurrent naive or anastomotic fibrostenosing Crohn's disease is efficacious and feasible.
Statement 17: The choice between surgery and repeated dilation in patients with naive or anastomotic fibrostenosing Crohn's disease should be made based on technical feasibility, symptom-free interval, patient preferences, remaining bowel length, length of fibrostenosing Crohn's disease, presence of inflammation at the site of the stricture and the location within the gastrointestinal tract.
Statement 18: In routine clinical practice, it is not recommended to treat naive or anastomotic fibrostenosing Crohn's disease with bare metal stents, anchored stents, removable stents, biodegradable stents or cutting techniques (for example, needle knife).

Table 5. Consensus statements on endoscopic treatment of fibrostenosing Crohn's disease[72]. *(Reproduced from Nature Reviews August 2024; D. Bettenworth et al)*

Statement 1: Strictureplasty should be the generally preferred option for anastomotic fibrostenosing Crohn's disease with lack of accessibility via endoscope.
Statement 2: The decision to perform strictureplasty in patients with naive or anastomotic fibrostenosing Crohn's disease should be based on the length of stricture, the presence of multiple strictures, the history of intestinal resection and the length of the remaining bowel.
Statement 3: The decision for the type of strictureplasty in patients with naive or anastomotic fibrostenosing Crohn's disease should be based on the length of stricture, the presence of multiple strictures, the history of intestinal resection and the length of the remaining bowel.
Statement 4: Intestinal resection should be the generally preferred option for naive fibrostenosing Crohn's disease with associated abscesses, phlegmon, internal penetrating disease, dysplasia, malignancy and long-segment fibrostenosing Crohn's disease.
Statement 5: Intestinal resection should be the generally preferred option for anastomotic fibrostenosing Crohn's disease with associated abscesses, phlegmon, internal penetrating disease, dysplasia, malignancy, lack of accessibility via endoscope and long-segment fibrostenosing Crohn's disease.
Statement 6: The laparoscopic approach in naive or anastomotic fibrostenosing Crohn's disease is preferable because of superior recovery, better cosmesis, fewer adhesions and incisional hernias, and similar surgical recurrence rates.
Statement 7: After successful surgical stricture resection of naive or anastomotic fibrostenosing Crohn's disease a structured follow-up strategy should include evaluation of obstructive symptoms, endoscopic evaluation and cross-sectional imaging of stricture recurrence.
Statement 8: After successful surgical stricture resection of naive or anastomotic fibrostenosing Crohn's disease the choice of anti-inflammatory therapy after surgery should depend on a thorough risk factor assessment.

Table 6. Consensus statements on surgical treatment of fibrostenosing Crohn's disease[72]**.** ***(Reproduced from Nature Reviews August 2024; D. Bettenworth et al)***

1.3: Pathogenesis of intestinal fibrosis

The pathogenesis of intestinal fibrosis is poorly understood, but often described to occur because of dysregulated wound healing in the susceptible patient[81].

The prior concept of fibrostenotic strictures resulting as an inevitable irreversible consequence of long-term inflammation in treatment refractory CD[82] patients has been challenged by recent advances in fibrogenesis research[31,83]. Nonetheless, no study has described fibrostenotic strictures occurring outside areas of inflammation[31,84].

In physiological wound healing, damaged tissue is restored to normal structure by controlled tissue repair mechanisms[85], while an uncontrolled and excessive response to tissue damage is characteristic in fibrosis[83].

In the presence of a transmural intestinal wound, fibroblasts, myofibroblasts (MF) and smooth muscle cells (SMC) accumulate in the site of the defect and secrete extracellular matrix (ECM) components, such as collagens and fibronectin to close the defect[86–88].

This process may be part of and which might help explain, the remarkable ability of the GIT for self-regeneration following short-lived and mild insults, as in infectious enteritis or mild diverticulitis[86–88].

Theoretically, in the context of chronic unrestricted, perhaps unregulated, inflammation, as observed in CD, the attempt to repair the resulting 'wound', an excessive production of ECM components results in the unintentional effect of luminal narrowing, stenosis and ultimately obstruction[31,89].

Whereas, wound healing mechanisms of fibrosis are observed and well described in other fibrotic disorders of the GIT[90] (in particular the liver[91,92] and pancreas[93]), the initiating factors and their continuing influences can often be identified[94]. Eventually, the resulting end organ insult and injury can sometimes lead to damaging fibrosis[94].

Contrastingly, this is an over simplistic analogy in the context of IBD: Firstly, there is no identifiable perpetuating pathogenic factor; and secondly the transmural fibrogenic reaction in CD is initiated and, to a limited extent, driven by, a granulomatous type of inflammation, which should be distinguished from an injury wound and subsequent healing process[95].

Once initiated, intestinal fibrogenesis is self-perpetuating and proceeds in the absence of inflammation, as evidenced by the minimal effect anti-inflammatory treatment has on fibrosis[81].

The proposed cascade resulting in fibrosis begins with tissue injury and chronic intestinal inflammation mediated by both innate and adaptive immune responses, and resultant release of profibrogenic molecules[96].

Unique to the GIT, multiple cell types may then become activated ECM-producing MF[97,98]. ECM production is then regulated by the balance between ECM degrading matrix metalloproteinases (MMP), and MMP regulating tissue inhibitors of metalloproteinases (TIMP), which ultimately determines whether appropriate tissue repair or fibrosis results. This balance is perturbed in the intestine of patients with fibrostenosing CD, where excess ECM production is a pathogenic feature of fibrosis (Figure 1)[30,99–101].

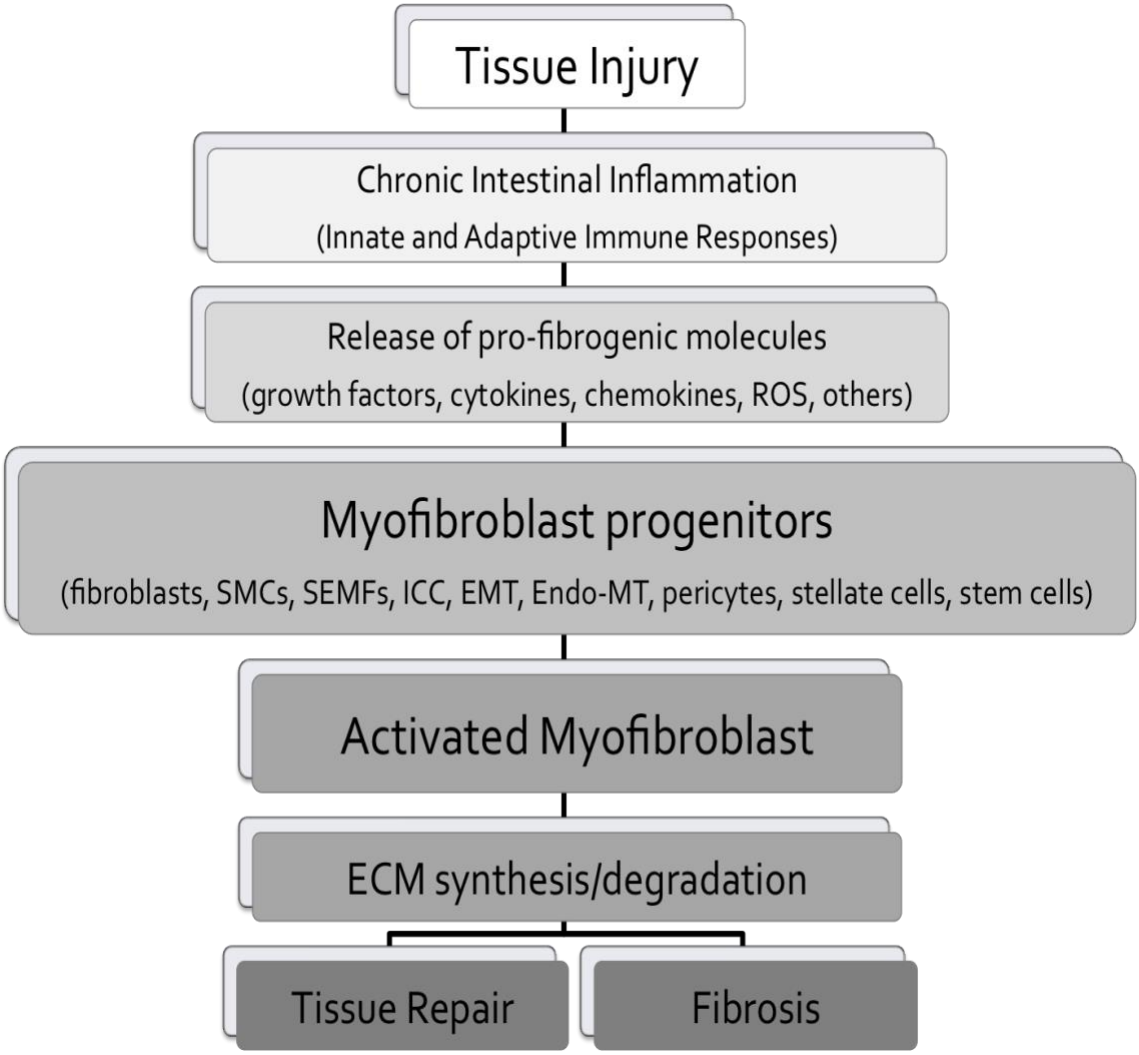


Figure 1. Pathogenic steps of intestinal fibrosis in CD. ROS: reactive oxygen species; SMCs: smooth muscle cells; SEMFs: intestinal subepithelial myofibroblasts; ICC: intestinal cells of Cajal; EMT: epithelial to mesenchymal transition; Endo-MT: endothelial to mesenchymal transition; ECM: extracellular matrix[58]. *(Modified from JCC October 2014; G. Latella et al)*

1.3.1: Immune response

Innate (genetically derived) and adaptive immune (insult driven) mechanisms are involved.

The Th2 and Th17-type immune responses observed in IBD are both profibrotic, with the former being defined by IL-4, IL-5 and IL-13 production. In contrast the Th1-type immune response, expressing interferon-γ (IFN-Y), has anti-fibrotic activity[58,102].

Considering fibrosis in general, it is appreciated that the communication between fibroblasts, macrophages, and CD4 T cells contribute to general and critical functions in initiating and perpetuating fibrosis, but also its resolution[102]. Although T cells activate fibroblasts and macrophages, their activation also starts negative feedback loops that limit the immune response to reduce fibrosis, of particular note when the Th2 cytokine IL-13 contributes to pathology[102].

In the presence of tissue damage, activated macrophages stimulate fibroblasts via various mediators, including transforming growth factor (TGF)-β, platelet derived growth factor (PDGF) and IL-1β, and promote inflammation by recruiting and activating monocytes and neutrophils, presenting antigens to CD4 T cells, and modulating T cell responses with co-stimulation and cytokines[102].

CD4 T cells coordinate the immune response, enhancing neutrophil recruitment with IL-17A, promoting macrophage activation with IL-4 and IL-13 or IFN-γ, and inducing collagen production by fibroblasts with IL-4, IL-13, and TGF-β[102].

Ultimately, fibroblast proliferation and activation and consequential synthesis of collagens, MMPs and TIMPs result in excess ECM production and deposition that is the hallmark of fibrosis (Figure 2)[102].

Conversely, phagocytosis of pathogenic cells or cellular material by activated macrophages, in addition to IL-10 meditated inhibition of profibrotic mediators, such as TGF-β, PDGF, and IL-1β, reverse MF activation. Activated macrophages can also moderate the CD4 T cell response, in addition to reducing fibrosis by producing nitric oxide[102].

Fibroblasts produce IL-13Rα2, a decoy receptor for IL-13, and contract to seal lesions[102].

Fibrosis then resolves as activated fibroblasts die or return to a quiescent state and macrophages degrade the excess ECM with MMPs[102].

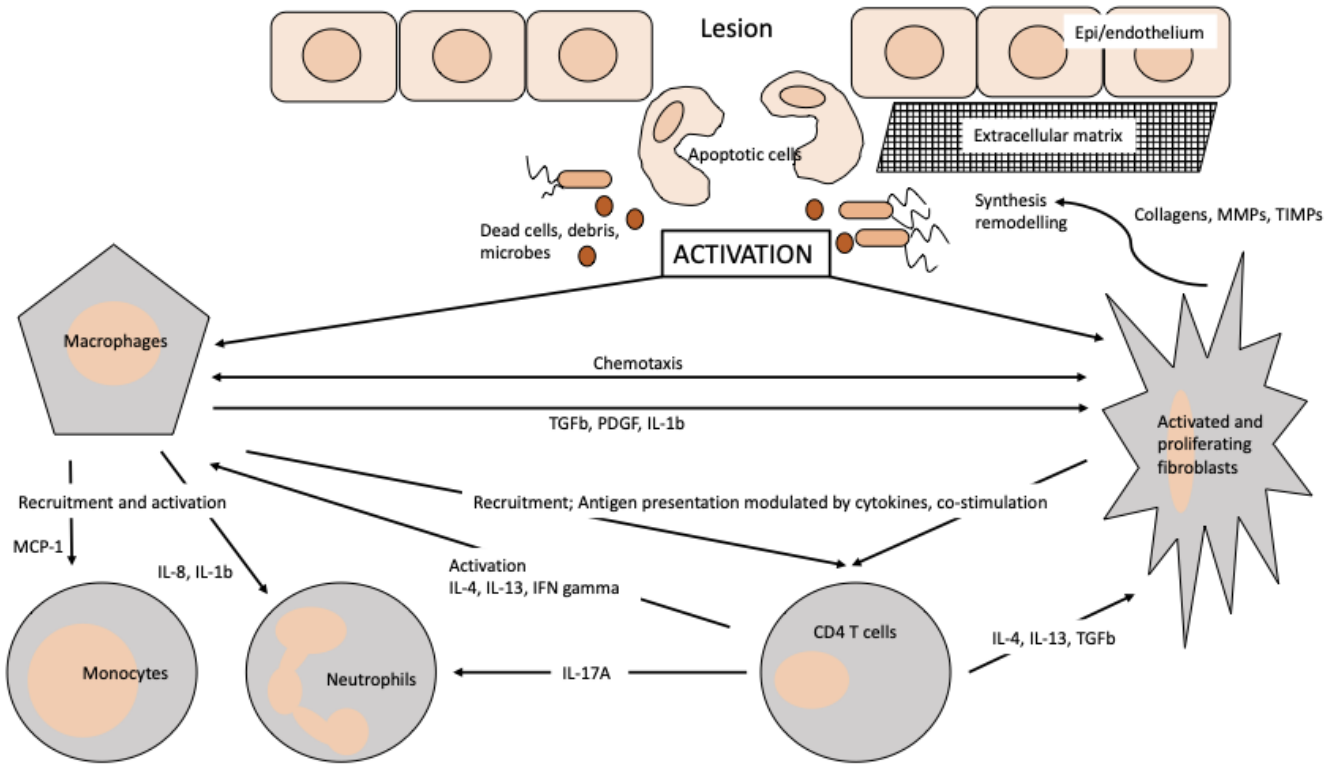


Figure 2. Model of the initiation and perpetuation of fibrosis. Injury activates macrophages and fibroblasts, enabling macrophages to simulate fibroblasts with TGF-β, PDGF, IL-1β, and other factors. Macrophages also promote inflammation by recruiting and activating monocytes and neutrophils, present antigens to CD4 T cells, and modulate T cell responses with co-stimulation and cytokines. Fibroblasts also influence T cells. CD4 T cells coordinate the immune response with cytokines, enhancing neutrophil recruitment with IL-17A, activating macrophages with IL-4 and IL-13 or IFN-γ, and inducing collagen production by fibroblasts with IL-4, IL-13, and TGF-β. The combination of activating signals from the inflammatory environment, macrophages, and CD4 T cells stimulate fibroblasts to proliferate and synthesize collagens, MMPs, and TIMPs that construct and remodel extracellular matrix and lead to fibrosis[102]. *(Modified from Am J Physiol Gastrointest Liver Physiol February 2011; L. Barron et al)*

1.3.2: Intestinal mesenchymal cell activation and derivation of myofibroblasts

Unlike fibrotic disease of other organs, sources of ECM-producing MF in the intestine are not restricted to a few cell types[97,98].

The mesenchymal cellular compartment can be expanded by the products of TGF-β1 driven Epithelial-Mesenchymal cell Transition (EMT), Endothelial-Mesenchymal cell Transition (Endo-MT) and invasion of bone-marrow derived circulating fibrocytes[81].

Intestinal injury results in the reprogramming of pluripotent epithelial and endothelial cells, which lose epithelial or endothelial markers and initiate synthesis of α-smooth muscle actin (α-SMA), experience cytoskeletal rearrangement, assuming a spindle shape, and migrate through the basement membrane from the compartment of origin to the interstitial space. Resultant production of ECM proteins, including collagen and fibronectin, is a significant contributor to the development of fibrosis[81].

Evolutionary processes involved include:

1. Through Endo-MT, endothelial cells lose their endothelial cell markers and acquire a mesenchymal phenotype, expressing α-SMA, vimentin, and collagen I[81].
2. Through EMT, resident tissue fibroblasts evolve from quiescent fibroblasts to cells expressing a MF phenotype[81].

3. From tissue migration of bone marrow-derived circulating fibrocytes, capable of producing fibroblastic proteins. These cells express α-SMA and collagen 1 characteristic of mesenchymal cells, and once recruited to sites of injury and inflammation differentiate into fibroblasts and MF, which not only respond to local profibrotic mediators, but also become sources of TGF-β1 and connective tissue growth factor (CTGF) to further cascade influence upon intestinal mesenchymal cells[81].

Each of these processes result in the presence of activated fibroblasts and MF within the intestinal wall at sites in injury[81].

Ultimately, chronic inflammation in CD and activation of the innate and adaptive immune systems results in release of cytokines and chemokines, elaboration of reactive oxygen species (ROS), release of pro-fibrotic cytokines and growth factors that result in activation of mesenchymal cells[81]. Once activated, their largely autocrine, profibrotic program can continue, even in the absence of continued inflammation, resulting in excess ECM deposition in the intestine, architectural distortion, and stricture formation (Figure 3)[81].

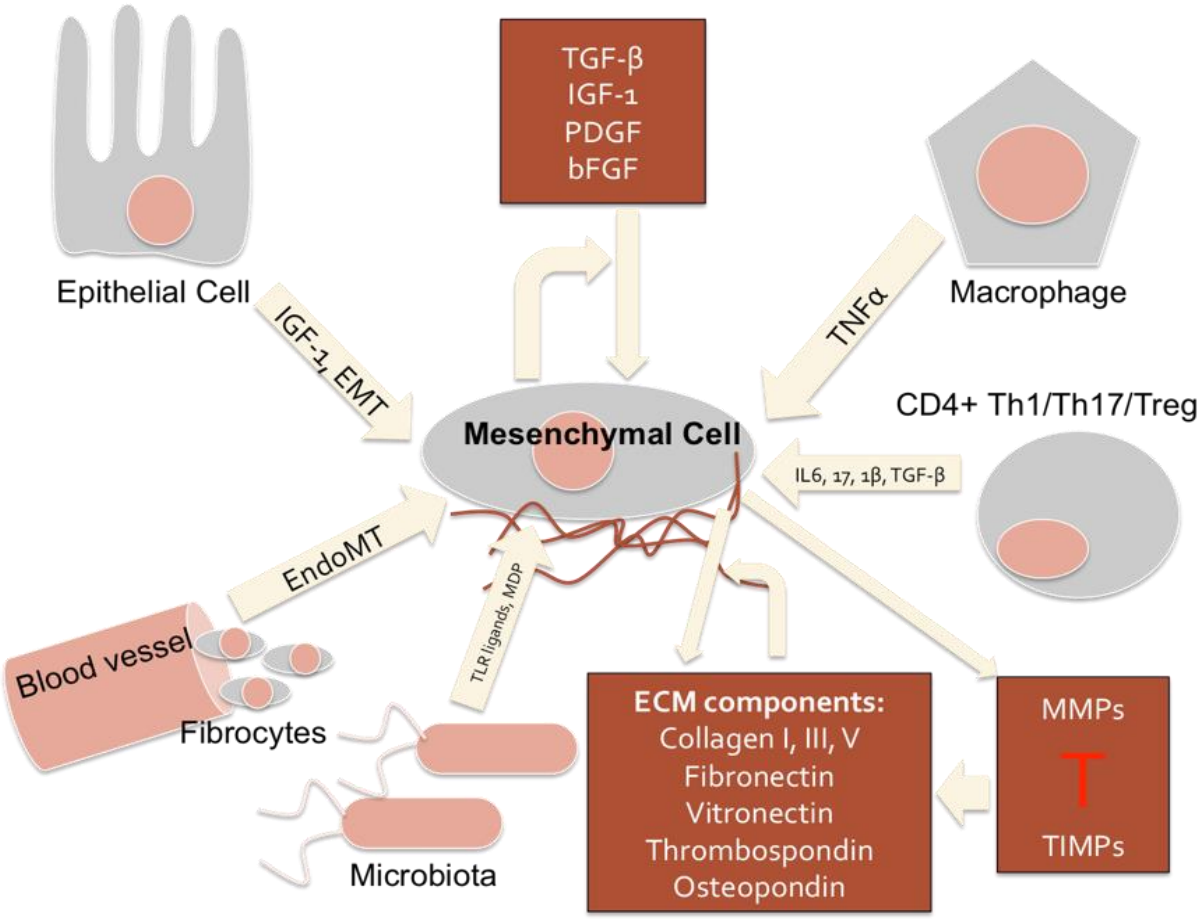


Figure 3. Activation of intestinal mesenchymal cells leading to fibrosis. TGF-β: transforming growth factor-β; IGF-1: inulin like growth factor-1; PDGF: platelet derived growth factor; bFGF: basic fibroblast growth factor; TNF-α: tumour necrosis factor-α; IL: interleukin; MMP: matrix metalloproteinase; TIMP: tissue inhibitors of metalloproteinases; TLR: toll-like receptor; MDP: muramyl dipeptide; Endo-MT: endothelial to mesenchymal transition; EMT: epithelial to mesenchymal transition[81]. *(Modified from IBD July 2014; C. Li et al)*

1.3.3: Activation, proliferation and regulation of myofibroblasts

Myofibroblast (characteristically α-SMA, vimentin, collage I positive[103]) activation and excess ECM production is a hallmark of intestinal fibrosis[81], and MF are distinctly phenotypically different in patients with Montreal B2 fibrostenotic disease compared to those with non-B2 disease. Specifically, the migratory potential of subepithelial MF from CD patients is dependent on the underlying disease phenotype. MF from patients with fibrostenotic disease exhibit decreased migration, whereas those from patients with penetrating disease exhibit increased migration[81,95].

Numerous molecular mediators have been implicated in the activation, proliferation and regulation of ECM-producing MF, which synergistically act to promote excessive fibrosis, rather than orderly wound healing[81,94,98].

Paracrine signalling by multiple cytokines, particularly IL-1β, IL-6, IL-13, and TNF-α; and growth factors, including TGF-β1, PDGF, CTGF, insulin like growth factor (IGF)-1 and -2, and basic fibroblast growth factor (bFGF), is observed[98,104,105].

Additionally, the highly synthetic activated MF are a rich autocrine source of many of these mediators[106].

Subepithelial MF also regulate the immune response. In normal intestine, they can regulate mucosal tolerance by supporting a FoxP3 positive regulatory T cell response, while in CD disease, MF are activated by the numerous paracrine

mediators in their environment that promote their proliferation and production of ECM. This has important implications on the crosstalk between subepithelial MF and epithelial cells, which respond to the paracrine stimuli from MF to increase their profibrotic TGF-β1 and TIMP-1 expression[81].

Separately and independently, luminal bacteria, almost certainly an important component of IBD aetiology, express Pathogen-associated molecular patterns (PAMPs), which activate immune and non-immune cells. PAMPs, including lipopolysaccharide (LPS), bacterial DNA and double-stranded RNA, bind to pattern recognition receptors, such as Toll-like receptors (TLR), triggering host defence, and activate immune and both pro-inflammatory and profibrotic gene expression[58,97].

TLR expression in non-immune cells is a key event leading to fibrosis, and increased expression of particularly TLR-3, -4, -6 and -7 is observed in CD, further promoting fibroblast differentiation into MF[107].

The renin-angiotensin system, specifically its principal effector, angiotensin II, is increased in CD intestine and influences all aspects of fibrogenesis, including cell growth, proliferation, differentiation, and apoptosis[108]. The production of TGF-β1 is strongly stimulated by the local activation of angiotensin II and mediates the fibrogenic response to injury. Contrastingly, angiotensin II blockade closely

correlates to a reduction of TGF-β1 expression, which can significantly improve or reverse fibrosis[108].

In trinitrobenzene sulphonic acid (TNBS)-induced chronic colitis-associated fibrosis model the angiotensin-converting enzyme inhibitor (ACEi), Captopril, was effective at preventing colorectal fibrosis and demonstrated that the antifibrotic action was mediated by a down-regulation of TGF-β1 overexpression[108,109].

1.3.4: Smooth muscle cells

Smooth muscle cells (characteristically α-SMA, desmin positive, collagen I positive), in addition to being an identifiable component of the resident intestinal mesencyhmal cell compartment [81], can differentiate or dedifferentiate towards a phenotype capable of producing large quantities of ECM proteins, particularly collagen I and III, fibronectin and vitronectin[110,111] and profibrotic cytokines and growth factors[81].

Three different subtypes of SMCs have been identified within a stricture using a combination of vimentin and two different antibodies that recognize α-SMA (1A4 and CGA7)[95]:

1. Highly differentiated SMC (vimentin negative, 1A4 positive CGA7 positive) located in the muscularis mucosa and propria, where there is marked thickening with cellular hyperplasia and hypertrophy, appear intimately involved in the development of fibrosis[95].
2. Moderately differentiated SMC (vimentin positive, 1A4 positive, CGA7 positive), are found along the borders between the submucosa and the two muscular layers[95].
3. Dedifferentiated SMC (vimentin positive, 1A4 positive, CGA7 negative) are occasionally found between the submucosa and the two muscular layers, infiltrating the submucosa[95].

The ubiquitous SMC in vascular pathology is postulated to be capable of switching towards a secretory phenotype with the ability to secrete ECM.

It remains unknown whether such a phenotype has implications in CD stricture development[95].

1.3.5: Fibroblasts

Fibroblasts (characteristically vimentin positive, collagen I positive, N-cadherin positive, but α-SMA negative) are a more heterogeneous group of cells than MF or SMC and are found scattered throughout the wall of intestine where they are involved in the response to injury, resulting in either normal wound healing or the development of fibrosis[81,95].

In similarity with MF and SMC, fibroblasts proliferate and produce ECM in response to TGF-β1, other growth factors such as IGF, and profibrotic cytokines including TNF-α[81].

In fibrostenotic CD N-cadherin expression is upregulated in response to TGF-β1 and increases fibroblasts migratory potential, in contrast to the reduced migration observed in subepithelial MF. This enhances infiltration of fibroblasts into the injured intestinal wall and this motility is further enhanced through autocrine production of the ECM protein, fibronectin[81,112].

The physical effect of ECM stiffening is itself capable of further activation of intestinal fibroblasts[81].

1.3.6: Transforming growth factor-β

Transforming growth factor-β [and the SMAD (see abbreviations) signalling pathway] is the central regulator in the injured and inflamed intestine, and while it has both wound healing and T-regulatory cell mediation properties, increased expression and activation is intrinsic to a core pathway of fibrosis (Figure 4)[81,98]. TGF-β exists in three isoforms: TGF-β1 and TGF-β3 expression is specifically increased in SMC, MF and fibroblasts of strictures; while TGF-β2 expression is increased in fibroblasts in the mucosal layer[81,113,114].

TGF-β is also involved in cellular differentiation, proliferation, transformation and immunoregulation[58].

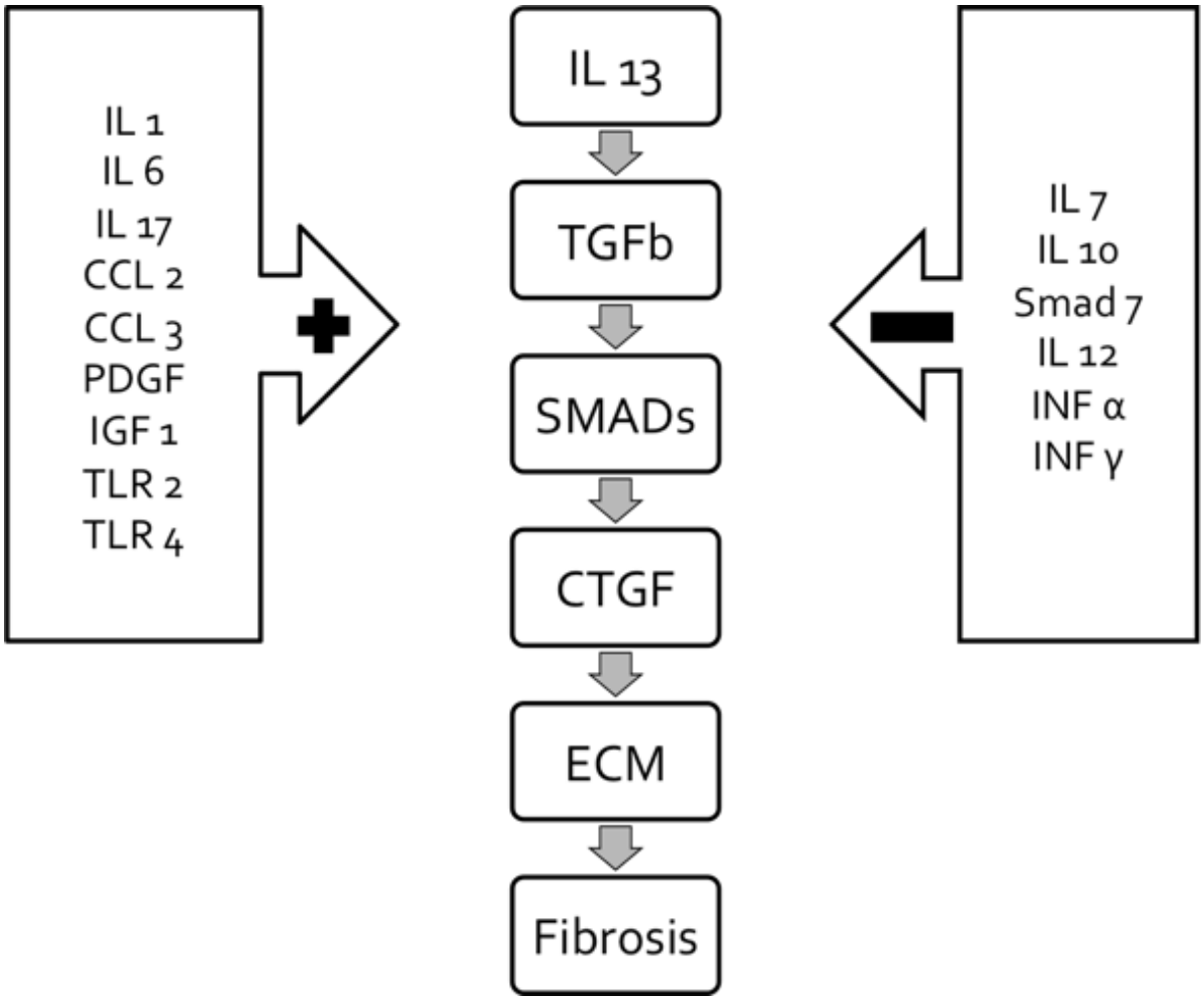


Figure 4. Relationship among several profibrotic and anti-fibrotic mediators in the development of fibrosis. IL: interleukin; CCL2: monocyte chemoattractant protein-1; CCL3: macrophage chemoattractant protein-1; PDGF: platelet derived growth factor; IGF-1: insulin like growth factor-1; TLR: toll-like receptor; TGF-β: transforming growth factor-β; CTGF: connective tissue growth factor; ECM: extracellular matrix; INF: interferon[58]. *(Modified from JCC October 2014; G. Latella et al)*

1.3.7: Extracellular matrix

Excess ECM production is a pathogenic feature of fibrosis, and once activated is regulated between the balance of MMPs that degrade ECM, and TIMPs that regulate MMP activity[30,99–101].

The ECM is comprised of structural proteins, particularly collagen I, III and V; specialised proteins, including vitronectin and fibronectin; and matricellular proteins, such as osteopontin and thrombospondin. Within gastrointestinal strictures there is increased expression of all these proteins[58,81].

If ECM deposition exceeds degradation, then fibrosis may occur[96,115]. Twenty-three (23) human zinc and calcium-dependent proteases, MMPs, can degrade all ECM proteins, which themselves are regulated themselves by four (4) TIMPs[116], and both are produced by epithelial, endothelial, and mesenchymal cells and macrophages. These are activated by proteolytic cleavage[30,81,99,117], but it is unclear in CD, however, which specific MMPs and TIMPs are involved in fibrosis and how they are regulated[58].

Known profibrotic cytokines and growth factors such as TGF-β and TNF-α can regulate MMP expression and promote fibrosis. In MF, TGF-β down-regulates MMP-1 and -3 expression, while enhancing TIMP-1 expression. TNF-α decreases MMP-2 activity and increases TIMP-1 expression[99].

1.3.8: Genetic associations

Whilst those patients who will develop fibrostenotic CD cannot be predictively identified, in this polygenic disease, patients carrying polymorphisms of certain genes, are more susceptible[118–120]. For example, the ATG16L gene, involved in autophagy, favour fibrosis; NOD2/CARD15 gene, responsible for bacterial muramyl dipeptide (MDP) recognition, is associated with aggressive, often fibrostenotic disease; and the cytokine receptor gene and member of the Th17/IL-23 pathway, IL-23R, stricturing ileal disease[121].

TLR variants, particularly TLR4, are associated with increased fibrostenotic small bowel CD[107,122]; TGF-β polymorphisms are associated with stricturing disease and shorter time to surgical resection[123]; and MMP-3 single nucleotide polymorphisms (SNP) increase the chance of stenotic complications[124].

1.4: Treatment modelling

Conventional IBD therapy has not made an impact on the incidence of stenotic complications in CD, and despite there being significant insight into fibrogenentic mechanisms, no clinically applicable anti-fibrotic treatment currently exists[58].

Many components of fibrogenesis including fibrogenic molecules, are considered potential therapeutic targets, but anti-fibrotic treatments remain at either a conceptual or early experimental stage[31,83].

A. Inhibition of TGF-β signalling would appear a logical strategy, however, attempted blockade of the TGF-β fibrotic pathway is problematic as TGF-β, SMAD2 and SMAD4 disruptions are lethal[31,58].

In animal models of fibrosis, deletion of TGF-β protein, its receptors, or SMAD proteins, other than SMAD 3, are generally lethal mutations or a feature of neoplastic cells[81].

Furthermore, and paradoxically, TGF-β has immune-regulatory functions, including inducing regulatory T-cells, and so suppression of which could exacerbate inflammation[125].

SMAD3 deletion mutants either developed a lethal wasting syndrome in association with immunodeficiency, or metastatic adenocarcinoma of the colon[126–128].

B. TGF-βRI/II receptors expressed by mesenchymal cells are linked to canonical SMAD2/3 signalling pathway[129]. Binding of active TGF-β1 to TGF-βRI/II receptors in human intestinal muscle activates canonical SMAD signalling, r-SMAD2/3 phosphorylation, and increased collagen I production that accounts for approximately 70% of collagen present in the intestine[110,129]. Monoclonal antibody inhibition of integrin αVβ6, involved in activation of latent TGF-β, reduce tissue levels of TGF-β1 and TGF-β2, αSMA, CTGF and TIMP1, and could potentially impact on fibrosis[98,130].

C. Resultant upon the renin-angiotensin system positively influencing fibrosis, the use of ACEi and angiotensin 1 receptor antagonists influence intestinal fibrosis, but not to therapeutic levels, apparently by reducing TGF-β1 and CTGF expression[108].

D. The trinitrobenzenesulfonic acid (TNBS) induced colitis is the best characterised murine model of inflammation-induced fibrosis, and increased expression of TGF-β1 and IGF-1 is observed. In this model, the non-specific cyclo-oxygenase (COX) inhibitor, Indomethacin, can be administered to induce intestinal inflammation and fibrosis, a process that can subsequently be decreased with treatment of prostaglandin E2 (PGE2)[58,131]. PGE2 significantly decreased ECM deposition by reducing TGF-β, collagen and TIMP expression, while increasing MMP expression[58,131].

E. A study in 2018 demonstrated that IL-17 inhibition significantly decreased profibrogenic cytokines and reduced fibrogenesis related TIMP-1 and MMP-2 gene expression in TNBS mice; however, previous clinical trials in CD patients with IL-17 monoclonal antibodies, secukinumab and brodalumab, only worsened disease activity[73,132].

F. Thalidomide has been shown to inhibit *in vivo* intestinal fibrosis by regulating TIMP/MMP protein balance and degradation of ECM[133,134].

1.5: Summary

Certainly, many key cells and profibrotic [and antifibrotic] signalling pathways that mediate them have been identified[58].

Simplistically, in CD, following initiative gastrointestinal inflammation, a predominantly TGF-β driven fibrotic cascade is triggered, affecting mesenchymal cell activation and cell differentiation to ECM-producing MF[81,95].

Excess, dysregulated ECM production, mediated by MMP/TIMP balance, results in fibrotic distortion of the intestine and stenosis[81,135].

At all points of this imbalanced pathway, synergistic effects and positive feedback, or the absence of negative feedback, only serve to exacerbate the fibrotic process and project this beyond the production of an innocent simple scar, into a damaging fibrotic mass[73,95].

However, it is clear this hypothesis does not satisfactorily translate to explain the whole clinical picture of CD or of the fibrostenosing phenotype of CD.

Firstly, and counter-intuitively, areas of severe luminal inflammation, if left uncontrolled, surprisingly do not consistently become fibrotic strictures[19,31].

Secondly, control of inflammation, should theoretically prevent fibrosis altogether, but rates of strictures remain unchanged, despite the increased

remission and maintenance of remission rates anti-inflammatory monoclonal antibody therapy has delivered[31].

At a clinical level there is a need to identify predictive biomarkers specific to fibrostenosing CD[136].

Identification of such parameters, at diagnosis or during the natural history of the disease, could determine tailored follow up management and more informed treatment decisions[137].

Such observations would inevitably stimulate the development and evaluation of anti-fibrotic medication.

2: AIMS AND OBJECTIVES

The overall objective of this research project is to contribute to the understanding of the mechanisms of intestinal fibrosis in CD:

1. Primarily by observing and determining differences in intestinal tissue from strictured and non-strictured areas resected from individual patients.
2. Observing and determining differences in isolated human MF harvested from both strictured areas and surrounding non-strictured areas of intestine resected from individual patients in Italy and donated to this study.

Given the current limited understanding of the CD fibrogenic process and its translation to the clinical phenotype[83], the project is aimed at providing a framework of information based on modern and accurate cellular methodology that will represent the basis for further mechanistic analysis.

Hypothesis 1:

Only intestinal MF isolated from strictured tissue will proliferate and demonstrate activation.

Aim 1: Identify differences in proliferation and activation of human intestinal MF from CD patients.

Hypothesis 2:

Intestinal MF isolated from fibrotic strictured tissue show higher fibrogenic activation than those assumed dormant MF isolated from adjacent non-strictured tissue.

Aim 2: Explore factors that may increase or suppress activation and migration of human intestinal MF obtained from strictured and non-strictured tissue from individual CD patients.

Hypothesis 3:

Relative expression of profibrotic genes will be higher in strictured than non strictured tissue.

Aim 3: Determine whether relative expression of known profibrotic genes in CD differs between tissue obtained from strictured and non-strictured areas of intestine resected from individual CD patients.

3: MATERIALS AND METHODS

3.1: Ethics

3.1.1: Myofibroblasts (donated from the University of Pavia)

Ethical approval was granted in Italy to isolate myofibroblasts (MF) from human Crohn's disease (CD) intestine in Pavia, Italy. Informed consent, obtained by local physicians, was obtained from identified potential patient donors prior to tissue collection from either surgical resection or endoscopic biopsy.

3.1.2: Tissue and Blood Collection at the Royal Free Hospital, London

For purposes of research detailed in the published protocol, "Exploring the Mechanisms of Intestinal Fibrosis in Stenotic Phenotypes of Crohn's Disease" (REC reference: 14/LO/1701; protocol number: 14/0244; IRAS project ID: 144029).

1. Ethical permission was obtained from London Riverside Ethics Committee, NHS Health Research Authority on 2nd December 2014.
2. Ethical permission was granted on 5th February 2016 for an additional amendment to (1).

Permitting the donation of tissue and blood samples from patients from patients with CD and from control patients without CD.

Informed consent was obtained from identified potential patient donors a minimum of 7 days after they had received the patient information sheet, and prior to tissue collection from either surgical resection or endoscopic biopsy.

3.2: Materials

3.2.1: Patients Studied

The following patients were recruited into this study:

Patient 1: Colonic MF samples from Italy – Montreal classification for location and behaviour **L2 B1**

Patient 2: Ileal MF samples from Italy – Montreal classification for location and behaviour **L2 B1**

Patient 3: Colonic samples from UK – Montreal classification **A2 L2 B2**, with prior anti-TNFα and thiopurine therapy exposure

Patient 4: Ileal and colonic samples from UK – Montreal classification **A3 L1 B2**, with prior anti-TNFα and thiopurine therapy exposure

Samples from these patients were not exposed to all experiments and are relevantly indicated in the described experimental protocols.

3.2.2: Human Myofibroblasts Source

Human MF were isolated from human ileum of one CD patient, and from a separate patient with colonic CD, from tissue obtained either from surgical or biopsies at colonoscopy.

Isolated MF were donated by Professor Antonio Di Sabatino, First Department of Internal Medicine, St Matteo Hospital, University of Pavia, Pavia, Italy, isolated using established published techniques[138,139].

Three separate subtypes of MF were isolated and cultured by colleagues in Italy. Suitable sample yields were then transported to our laboratory frozen on dry ice (minus 78.5°C), and subsequently vials of cells stored in liquid nitrogen.

The following methodologies describe below identify the standardised methods of native MF isolation and validation in Italy prior to dispatch.

All subsequent methodologies are those which were performed in our UK laboratory by me upon thawing cells to produce workable, reliable yields of MF for study.

3.2.3: Human Myofibroblast Subtypes (Italy)

The CD patients from whom the MF were isolated had the following phenotypes (Table 7):

Patient 1: **CDhuIC-4 MF (Sample 1)** were isolated from inflamed, non-strictured colonic mucosa.

Patient 2: **CDhuUI-2** and **CDhuSI-2** MF were obtained respectively from specimens of two separate locations from this single patient with stricturing small bowel CD.

CDhuUI-2 MF (Sample 2) from uninflamed, non-strictured ileal mucosa, and **CDhuSI-2 MF (Sample 3)** from established strictured ileal mucosa.

Myofibroblast group	**Assigned nomenclature**	**Location and macroscopic appearance of intestinal mucosa**	**Patient Montreal classification for location and behaviour**
Sample 1 (Patient 1)	CDhuIC-4	Inflamed, non-strictured colon	L2 B1
Sample 2 (Patient 2)	CDhuUI-2	Uninflamed, non-strictured ileum	L1 B2
Sample 3 (Patient 2)	CDhuSI-2	Uninflamed, strictured ileum	

Table 7. Isolated myofibroblast nomenclature and corresponding patient phenotype.

3.2.4: Myofibroblast Isolation (Italy)

Methodological details of standardised isolation form tissue specimens obtained in Italy[138,139].

For each specimen the mucosa was removed from other layers of the tissue sample; cut into 3mm fragments; washed with HBSS to remove mucus; and incubated with agitation in 10 ml 1 mM EDTA plus 1 mM HBSS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 10 minutes.

The mucosa fragments were then further dissected into smaller pieces and incubated with agitation in 20 ml of Gibco® by Life Technologies Dulbecco's Modified Eagle Medium (DMEM), supplemented with 20% (100 ml in 500 ml DMEM) foetal bovine serum (FBS), 5 ml [in 500 ml DMEM] of Sigma Life Science Penicillin-Streptomycin (10,000 units-10 mg), 0.6 ml [in 500 ml DMEM] of Sigma Life Science Gentamicin solution (50 mg/ml), 1mg/ml collagenase Ia (0.2 µm filtered), and 10 U/ml DNase I at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 1 hour.

Subsequently, the mucosal fragments and medium were mechanically disrupted repeatedly with a syringe and passed through a 100 µm cell strainer into a 50 ml Falcon tube; extra medium added as necessary to achieve a final volume of 40 ml; and centrifuged at 1500 rpm at 4°C for 10 minutes.

The resulting pellet was re-suspended in 1 ml pre-warmed Gibco® by Life Technologies Dulbecco's Modified Eagle Medium (DMEM), supplemented with 20% (100 ml in 500 ml DMEM) foetal bovine serum (FBS), 5 ml [in 500 ml DMEM] of Sigma Life Science Penicillin-Streptomycin (10,000 units-10 mg), 0.6 ml [in 500 ml DMEM] of Sigma Life Science Gentamicin solution (50 mg/ml), and cell (lamina propria mononuclear cells [LPMC]) count performed.

Identified LPMCs were seeded in 25 cm^2 flasks (2×10^6 cells/flask) and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cells were checked at 24-hour intervals, and medium changed if and when MF adhered to the flask.

The MF culture cycles were repeated to the satisfaction of the Italian group in the provision of sufficient workable material for transportation to the UK on dry ice (minus 78.5°C).

3.3: Methods

3.3.1: Myofibroblast culture and characterisation (Italy and UK)

Methodological details of the culture of isolated MF performed in Italy at initial harvest and subsequently in the UK for yield gains on thawed samples.

The obtained MF were cultured in Gibco® by Life Technologies Dulbecco's Modified Eagle Medium (DMEM), and 500 ml volumes were supplemented with 100 ml FBS (20%), 5 ml [1%] of Sigma Life Science Penicillin-Streptomycin (10,000 units-10 mg), and 0.6 ml [0.12%] of Sigma Life Science Gentamicin solution (50 mg/ml), constituting and subsequently referred to as complete medium (CM).

MF were cultured under standard conditions at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The CM was routinely renewed 3 times a week, and cells were tested regularly for any mycoplasma contamination employing Polymerase Chain Reaction (PCR) and performed in by the Institute for Liver and Digestive Health at the Royal Free Hospital and Italy.

In Italy passaging was performed to provide that considered sufficient for delivery to the UK and further culture locally.

The harvested MF were subjected to characterisation and validation in Italy using the following antibodies: Anti–α-smooth muscle cell actin (clone 1A4;

DAKO, High Wycombe, UK), antivimentin (clone V9; Santa Cruz Biotechnology, Wiltshire, UK), anti-PR2D3, antidesmin (clone D33; DAKO), anti–cytokeratin-18 (clone CY-90; AbCam, Cambridge, UK), anti-CD3 (clone UCHT1; DAKO), anti-CD68 (clone PG-M1; DAKO), and appropriate isotype-matched controls (Sigma-Aldrich)[140].

In the UK, following thawing and culture of the native MF samples from Italy, experiments were performed on locally harvested MF between passages 7 and 9.

3.3.2: Myofibroblast Morphology (UK)

Myofibroblasts cultured in CM were photographed using white light microscopy magnification to assess and describe basic morphology[141].

Myofibroblasts share morphological features with conventional tissue fibroblasts and contractile SMC, which are found in the colonic mucosa. MF and fibroblasts are spindle shaped cells with a flattened oval nucleolus often with long thing cytoplasmic extensions[142].

3.3.3: Myofibroblast Proliferation and Activation (UK)

Proliferation:

To establish a baseline comparison of the behaviour of the three distinct samples, MF were cultured in either CM or serum free medium (SFM) for 48 hours and differences in proliferation were assayed using Bromodeoxyuridine (BrdU) incorporation[143,144].

Bromodeoxyuridine (BrdU) incorporation assay

Cell proliferation was quantified by BrdU Cell Proliferation ELISA kit (Roche). The assay is based on the replacement of thymidine by the pyrimidine analogue bromodeoxyuridine (BrdU) in proliferating cells. A colorimetric immunoreaction as a measure of BrdU incorporation into newly synthesized cellular DNA can be detected photometrically (Omega Plate Reader, absorbance at 370 nm)[143,144].

Activation:

Parameters of activation, following incubations either for 24 and 48 hours were analysed using Western blot analysis of the established protein markers; α-SMA, ERK and phosphorylated ERK expression[88,141].

Western blot

Western blot requires the collection of a cell lysate of the culture, which can be assessed for protein content. Successful samples are then subjected to SDS-PAGE electrophoresis[145].

Separated products of the electrophoresis, can then be exposed to specific target marker antibodies with marker-antibody complexes then being identified by generic antibody counterstaining[145].

Total protein cell lysate

Harvested cultured cells, obtained by scraping the flask wall, were washed with 1x phosphate buffered saline (PBS), then, following the addition of total protein lysis buffer (RIPA: 20mM Tris-HCl pH7.6, 150 mM sodium chloride, 5 mM EDTA, 1% NP-40 (nonylphenoxypolyethoxylethanol), 1mM phenylmethylsulfonyl fluoride (PMSF), 1x Protease Inhibitors Mix, 1mM Na_3VO_4, and 1mM sodium

fluoride), the cell harvest was sonicated with the 'Ultrasonic Processor' (Sonics, Vibra-Cell) at an amplitude of 50, centrifuged at 11,000 rcf at 4°C for 10 minutes. This treatment produced lysate which was then used or stored at minus 80°C.

Protein quantification

Protein concentrations were assayed using, a bicinchoninic acid (BCA).

The assay was used according to manufacturer's protocol (Micro BCA™ Protein Assay Kit, Thermo Scientific), and is a method identifying the formation of a purple-coloured reaction product, determined by the reduction of Cu^{2+} ions by present protein. The reaction product exhibits absorbance at 562 nm, which correlates linearly with increasing protein concentration (Fluostar Omega Plate Reader [BMG Labtech]).

SDS-PAGE

To separate proteins, protein lysates prior to loading onto gel substrate, were supplemented with 4x Laemmli Sample Buffer (Bio-Rad) containing 5% β-mercaptoethanol and boiled.

Subsequently, 25 µg aliquots of protein lysate were loaded on 10% acrylamide gels (<u>separating gel:</u> 10% acrylamide mix, 0.4M Tris [pH 8.8], 0.1% SDS, 0.1%

ammonium persulfate, 0.001% TEMED, H_2O; stacking gel: 5% acrylamide mix, 0.1M Tris [pH 6.8)], 0.1% SDS, 0.1% ammonium persulfate, 0.001% TEMED, H_2O). An exception to this protocol, was employed for protein lysates used for collagen 1 expression analysis. In these specific experiments, where non-denatured, non-reduced protein provided optimal results, β-mercaptoethanol was not added and samples were not boiled.

Electrophoresis was performed using, 100-150V applied for 90-150 minutes. Mini PROTEAN® System (Bio-Rad) was used with 1x Running Buffer (Ultra-Pure 10x Electrophoresis Grade, 0.25M Tris, 1.92M Glycine, 1% SDS, GeneFlow). Precision Plus Protein™ Standards Dual Colour (Bio-Rad) was employed as protein standard control run.

To verify equal loading of samples, aliquots of tubulin or vinculin were added and subsequently identified synchronously by later antibody detection.

Separated proteins were blotted from the gel to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P Transfer Membranes, Millipore™) by wet transfer at 100V for 75 minutes with a transfer buffer containing 25mM Tris, 192mM Glycine and 10% Methanol.

The PVDF membranes were stained pink with Ponceau S solution (0.1% (w/v) in 5% acetic acid, Sigma) to visually verify the successful transfer of protein indicated by the development of a pink colour.

Antibody incubation

Membranes were blocked with 5% Bovine Serum Albumin (BSA, Sigma) in Tris Buffered Saline with 0.1% Tween-20.

Primary targeting antibodies were incubated overnight at 4°C, or for 1 hour at room temperature.

After washing, specific secondary antibodies coupled with horseradish peroxidase were applied for 1 hour at room temperature.

Following incubation, SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) was used to develop signals from control markers and test sample.

To facilitate the investigation of more than one target protein on a single blot each specific antibody was stripped with Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific) between antibody incubations.

Antibodies are listed in table 8.

Antibody	Species	Company
Alpha-smooth muscle actin ab5694	Rabbit	Abcam
p44/42 MAPK (Erk 1/2) (137F5) ♯4695	Rabbit	Cell Signaling Technology
Phospho-p44/42 MAPK (Erk 1/2) (Thr202/Tyr204) (D13.14.4E) XP™ ♯4370	Rabbit	Cell Signaling Technology
α-Tubulin ♯2144	Rabbit	Cell Signaling Technology
Collagen 1 NB600-408	Rabbit	Novus Biologicals
Anti-Vinculin [EPR8185] ab129002	Rabbit	Abcam

Table 8. Primary antibodies for western blot analysis.

Subsequently, further proliferation and/or activation experiments were also performed using these same common techniques on every occasion MF subtypes were cultured in differing incubation conditions.

3.3.4: Preparation of Conditioned Medium

Conditioned medium (CndM) is the resultant culture medium obtained following a period of culture of an MF subtype in SFM. This medium is retained in the likelihood of it containing components secreted by the incubated MF as previously demonstrated by our laboratory[146–149].

Conditioned medium of an individual MF subtype was obtained by culturing 0.3 x 10^6 MF in 35 mm culture wells in CM.

After 24 hours, cells were washed three times with HBSS and incubated in serum-free medium (SFM) for 48 hours.

Conditioned medium was collected by centrifugation at 1000 rpm at 21°C for 10 minutes to remove cellular debris.

3.3.5: Cross Incubation Experiments

These experiments were designed to investigate the possibility of paracrine mechanisms operating by soluble secreted cell products within the culture medium[150,151].

Each MF subtype was cross cultured in, conditioned medium (CndM) obtained from each of the other MF subtypes (Table 9).

MF were plated on a cell culture dish (6 wells) in CM and after 24 hours were washed with HBSS and culture continued in SFM for a further 24 hours.

These serum starved cells were then each incubated with CndM of opposing MF subtypes, but in only 4 of the 6 wells.

Two of the 6 wells were controls where in each, the serum starved MF were incubated with fresh CM (positive active control) or SFM (negative control).

MF activation and proliferation was assessed after 48 hours.

Myofibroblast cell subtype	**Conditioned culture medium derived from:**	
Sample 1 (CDhuIC-4)	Sample 2 (CDhuUI-2)	Sample 3 (CDhuSI-2)
Sample 2 (CDhuUI-2)	Sample 1 (CDhuIC-4)	Sample 3 (CDhuSI-2)
Sample 3 (CDhuSI-2)	Sample 1 (CDhuIC-4)	Sample 3 (CDhuSI-2)

Table 9. Cross incubation with conditioned media.

3.3.6: Treatment of Myofibroblasts with TGF-β1

To further investigate factors involved in activation of MF specifically obtained from different areas of small bowel, MF from both sample 2 (CDhuUI-2) and sample 3 (CDhuSI-2) of the patient with a stricturing phenotype of CD, were cultured in SFM but separately containing two different active concentrations (2ng/mL and 10ng/mL) of the known profibrotic mediator TGF-β1[152,153] (R&D systems, Minneapolis, MN, USA).

These experimental concentrations of TGF-β are known to exert a profibrotic effect in hepatic stellate and intestinal cells[154,155].

MF were plated on a cell culture dish (6 wells) in CM and after 24 hours were washed with HBSS and culture continued in SFM for a further 24 hours.

These MF subtypes were then separately in parallel, exposed to two concentrations of TGF-β (2ng/ml and 10ng/ml), prepared in SFM in 4 of the 6 wells.

Two of the 6 wells were controls where in each the serum starved MF were incubated with fresh CM (positive active control) or SFM (negative control).

After 24 hours incubation protein extraction experiments were performed to assess only MF activation.

3.3.7: Collagen 1A Analysis

Collagen 1A (COL1A) could be an expected product found in synthetically active MF[156,157].

Western blot was performed to explore any expression of COL1A, a major structural protein of the extracellular matrix[58,155].

Samples used (sample 2 [CDhuUI-2] and sample 3 [CDhuSI-2]) were from patient 2 with the fibrotic phenotype of CD, and incubated with CM and SFM and exposed to TGF-β at the two concentrations of 2 and 10 ng/ml.

3.3.8: Wound Scratch Assay

A wound scratch assay was performed to assess differences in migration[158] of the two MF subtypes, CDhuUI-2 (sample 2) and CDhuSI-2 (sample 3).

Following initial culture, the cell monolayer on each 6 well plate was scraped with a p200 pipette tip to create a 'wound ' or 'scratch'. Cells were washed in the corresponding culture medium to remove debris and the specific medium then replaced[159].

Migration of MF into the wound was simultaneously assessed at 24 hours and 48 hours post scratch using white light microscopy magnification under certain conditions:

1. On MF cultured in standard conditions (CM and SFM).
2. On MF harvested from cross cultured incubations as previously described section 3.3.5.
3. On MF incubated in medium treated with 2 ng/ml TGF-β.

3.3.9: RNA Isolation

In a completely separate series of experiments performed on tissue from patient 3, macroscopically uninflamed samples from strictured and non-strictured colonic mucosa were obtained by biopsy at colonoscopy.

Two samples from macroscopically normal mucosa (site numbers 1 and 2) were taken in addition to two samples from an established colonic stricture (site numbers 3 and 4). (Table 10).

RNA was isolated from these samples and was then analysed for relative expression of genes known to influence fibrogenesis.

Clinically, polymorphisms in NOD2 and IL-23R genes have well documented associations with aggressive fibrostenotic disease, particularly ileal and

stricturing disease respectively[121]. It is not known whether this patient has polymorphisms for NOD2 or IL-23R.

Chosen probes spanned exons known to be devoid of possible naturally occurring single nucleotide polymorphisms (SNPs company information), it was considered important and worthwhile to assess relative mRNA expression of these genes.

	Location and macroscopic appearance of intestinal mucosa of Patient 3
Site 1	Colon – non-strictured, uninflamed
Site 2	Colon – non-strictured, uninflamed
Site 3	Colonic stricture – uninflamed
Site 4	Colonic stricture – uninflamed

Table 10. Colonic biopsy nomenclature and sample location appearance.

RNA was isolated using the RNeasy mini Kit (Qiagen) according to the manufacturer's protocol. Purity and RNA concentration were measured with NanoDrop spectrophotometer (Thermo Scientific)[160].

Complementary DNA (cDNA) was synthesised with MultiScribe reverse transcriptase, random primers, deoxyribose nucleoside triphosphate (dNTP) mix and RNase inhibitor (Applied Biosystems).

3.3.10: Gene Expression – Quantitative real-time PCR

Gene expression was measured by quantitative real time PCR (qPCR) using TaqMan® gene assays (Applied Biosystems) (Table 11)[161]. The comparative C_T method was employed to quantify gene expression[162], using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control.

Gene	Assay Identification	Company
GAPDH	Hs02758991_g1	Applied Biosystems
NOD2	Hs00223394_m1	Applied Biosystems
IL-23R	Hs00332759_m1	Applied Biosystems
COL1A1	Hs00164004_m1	Applied Biosystems
COL3A1	Hs00943809_m1	Applied Biosystems
LOX	Hs00942480_m1	Applied Biosystems
TNF-α	Hs01113624_g1	Applied Biosystems
TGF-β	Hs00998133_m1	Applied Biosystems
ACTA2	Hs00426835_g1	Applied Biosystems
TIMP-1	Hs00171558_m1	Applied Biosystems
MMP-2	Hs01548727_m1	Applied Biosystems
MMP-9	Hs00234579_m1	Applied Biosystems
IL-6	Hs00985639_m1	Applied Biosystems
IL-1B	Hs01555410_m1	Applied Biosystems
CCL2	Hs00234140_m1	Applied Biosystems
IL-8	Hs00174103_m1	Applied Biosystems
IL-12B	Hs01011516_g1	Applied Biosystems

Table 11. TaqMan® gene assays used for real-time PCR.

3.3.11: Myofibroblast Isolation in this UK Laboratory – primary attempt

Tissue was obtained from six sites across a surgically resected terminal ileal stricture in patient 4. Each site was described by macroscopic appearance and location (Image 2):

Site 1: Uninflamed ileum.

Site 2: Inflamed ileum.

Site 3: Inflamed ileum.

Site 4: Strictured terminal ileum.

Site 5: Uninflamed caecum.

Site 6: Uninflamed ascending colon.

Myofibroblast isolation was attempted from each specimen using the previously described methodology (see section 3.2.4).

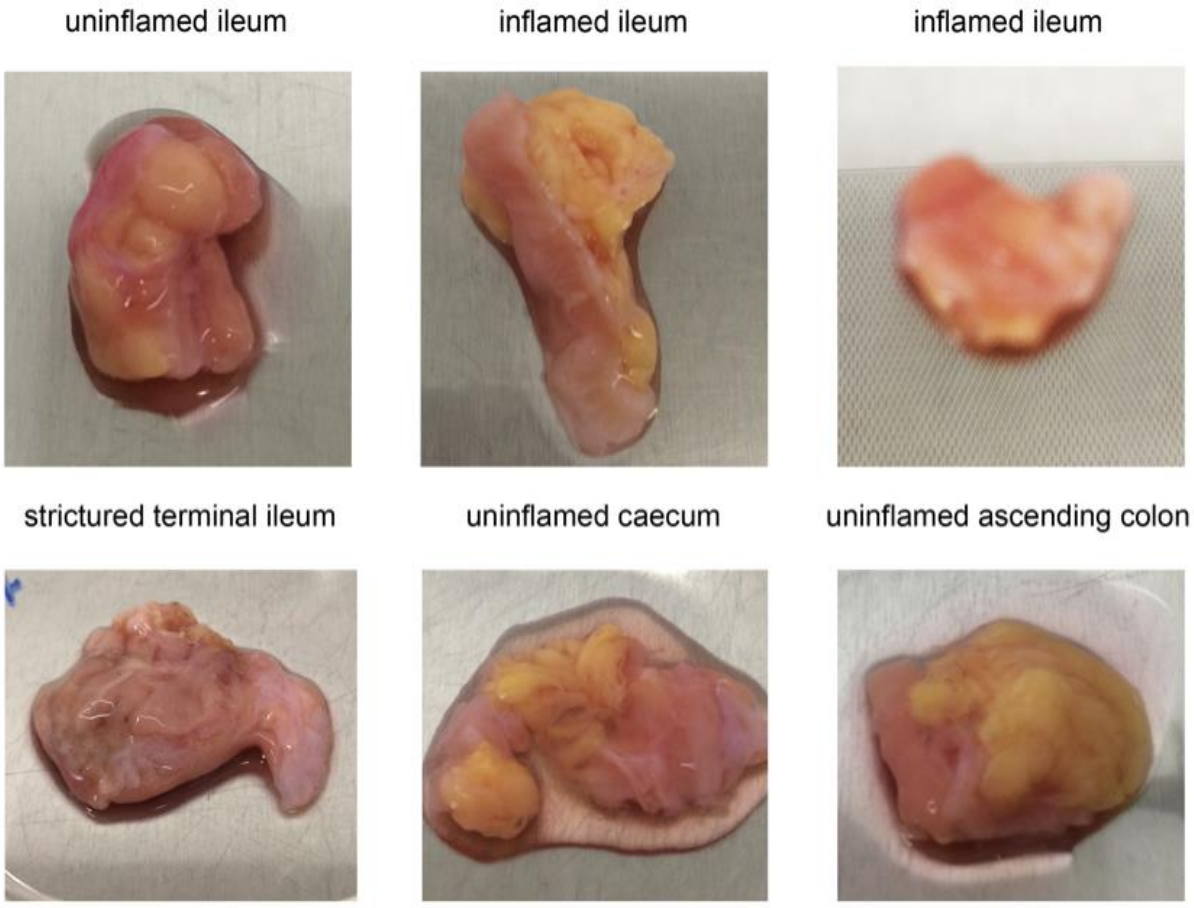


Image 2. Surgical resection specimens from a patient with fibrostenotic Crohn's disease.

3.4: Statistical Analysis

Statistical analysis was performed using Microsoft Excel or Graph Pad Prism. Values are expressed as, mean +/- standard deviation (SD); mean +/- standard error of the mean (SEM); or mean +/- 95% confidence interval. Statistical significance was analysed with unpaired, parametric Student T-test.

4: RESULTS

The following set of experiments were designed to investigate a series of biological properties of the isolated MF which included, morphology, proliferation, activation and migration.

4.1: Myofibroblast Isolation at the Royal Free Hospital Laboratory

We attempted to isolate MF from locally obtained specimens using the established protocol described by Antonio de Sabatino's group[140].

Attempted MF isolation and culture from the resected specimens of Patient 4 was unsuccessful at this first ever attempt at the Institute for Liver and Digestive Health, Royal Free Hospital London.

This was not repeated within the time frame of this submission.

4.2: Studies on the Isolated Myofibroblasts

4.2.1: Myofibroblast Morphology

White light microscopy confirmed recognised morphological features of isolated MF[142].

Each MF subtypes cultured from Patients 1 and 2 demonstrated morphological differences.

CDhuIC-4 MF (colonic, patient 1) show a typical myofibroblast-like phenotype. (Image 3A)[141].

Both CDhuSI-2 and CDhuUI-2 MF derived from small bowel (ileal, patient 2), demonstrated features of fibroblasts. (Images 3B and 3C)[141].

It was observed that populations of MF from different parts of the bowel have different morphology, with those from the small bowel more closely resembling the mature fibroblast. This might indicate that MF from differing parts of bowel were exposed to differing biological pressures.

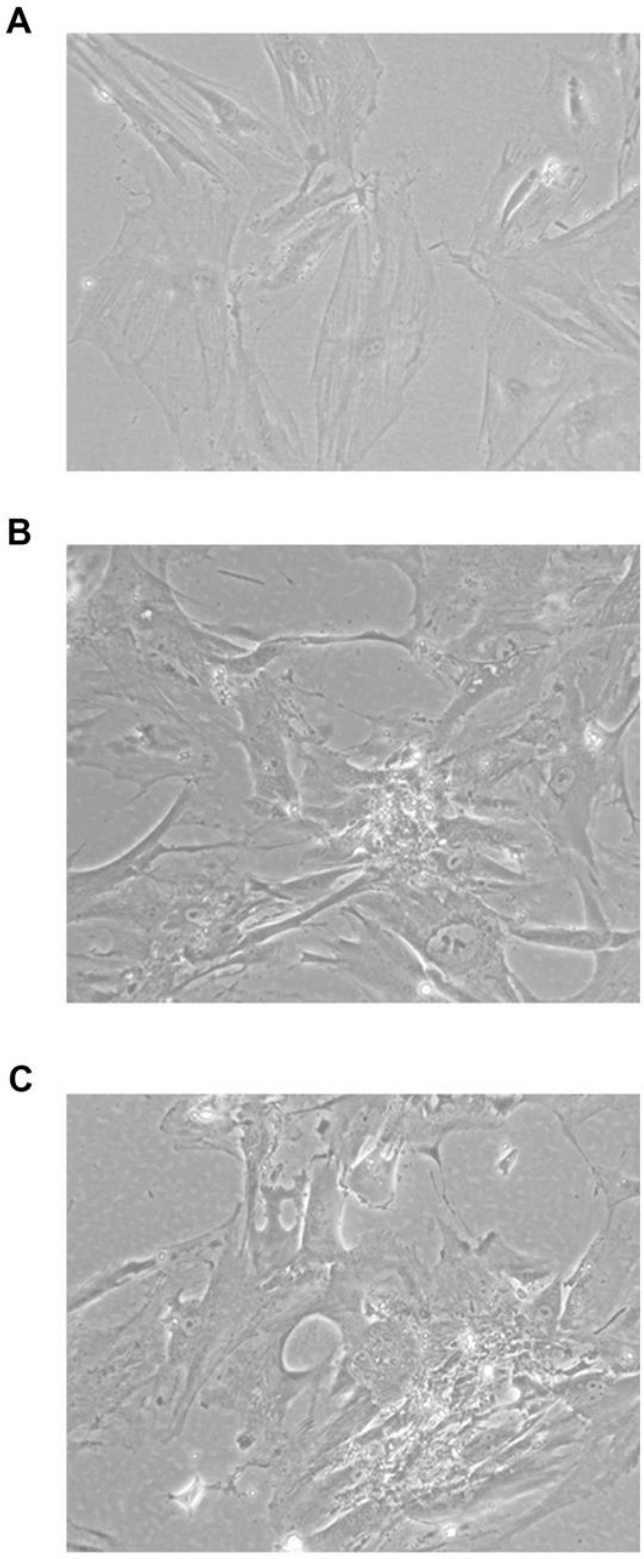


Image 3. Morphological differences between intestinal myofibroblasts derived from (A) Crohn's disease inflamed colon (patient 1, CDhuIC-4), (B) Crohn's disease uninflamed strictured ileum (patient 2, CDhuSI-2), and (C) Crohn's disease uninflamed non-strictured ileum (patient 2, CDhuUI-2).

4.2.2: Myofibroblast Proliferation

It is known that cell proliferation can differ when cultures are exposed to a serum containing medium compared, for example, to a serum free medium.

It is accepted that serum free media does offer greater experimental consistency than serum containing media when cellular growth and proliferation can be supported without the need for serum[163,164].

A small series of experiments that would complement the morphological observations were designed to interrogate whether the cultured MF would proliferate and demonstrate any differences between cells isolated from different bowel localities.

Complete and Serum Free Media Incubations

Each subtype of MF persistently had significant increased proliferation when cultured in complete medium (CM) compared to serum free medium (SFM) where less proliferation was observed (Figure 5).

These observations were used as necessary for experimental controls.

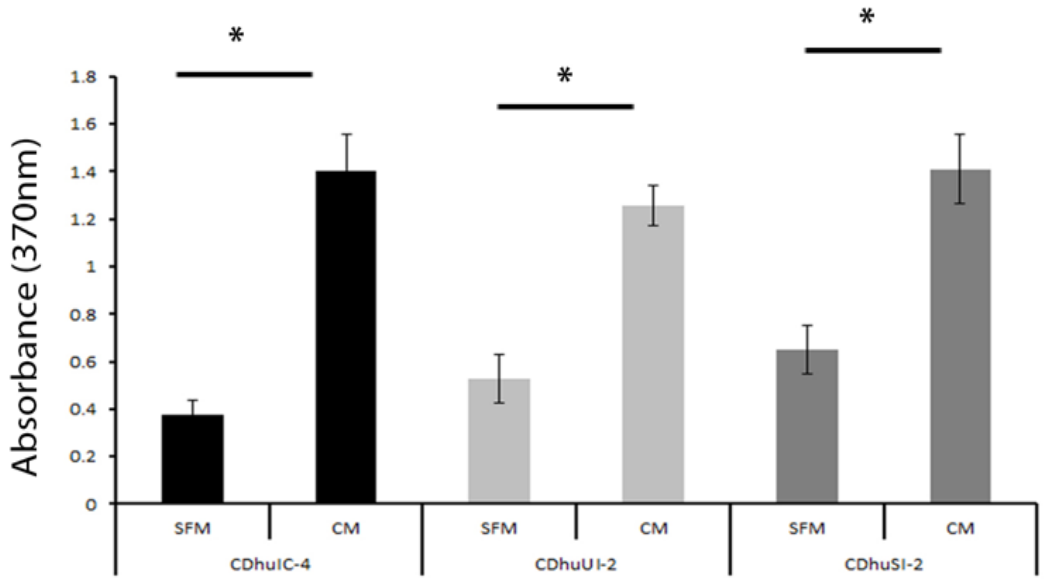


Figure 5. Proliferation of myofibroblasts from distinct areas of Crohn's disease activity. MF significantly ($p < 0.05$) proliferate more in complete culture medium (CM) compared to serum free medium (SFM).

MF subtype from strictured ileum (CDhuSI-2, sample 3), demonstrated statistically significant increased proliferation compared to those from both uninflamed, non-strictured ileum (CDhuUI-2, sample 2), and from inflamed colon (CDhuIC-4, sample 1).

Those MF from inflamed colon did proliferate, but statistically significantly less than both MF subtypes derived from ileal tissue (Figure 6).

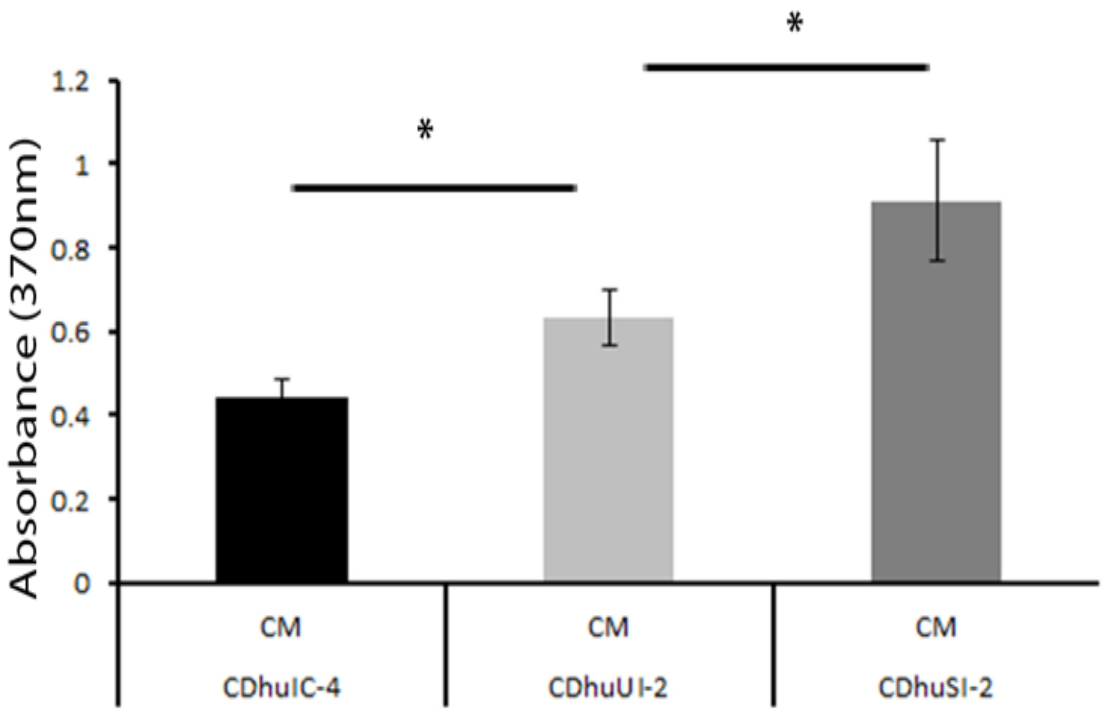


Figure 6. Myofibroblasts from strictured ileum demonstrated significant ($p < 0.05$) increased proliferation compared to those from both uninflamed, non-strictured ileum and inflamed colon.

Conditioned Media Incubations (Cross Culture) – Proliferation

These experiments were designed to observe any influence of the soluble phase culture medium taken from one set of cultured MF subtype on the behaviour of another MF subtype exposed to this crossover and possibly conditioned medium.

Although MF from distinct areas of Crohn's disease activity significantly proliferate more in complete medium compared to serum free medium, and MF from strictured ileum demonstrated significant increased proliferation compared to those from both uninflamed, non-strictured ileum and inflamed colon, MF proliferation is not significantly altered when cultured in conditioned media.

No significant change of either induction or suppression of proliferation of any incubation combination was observed in any MF subtype and was comparable to the negative control (SFM). (Figure 7).

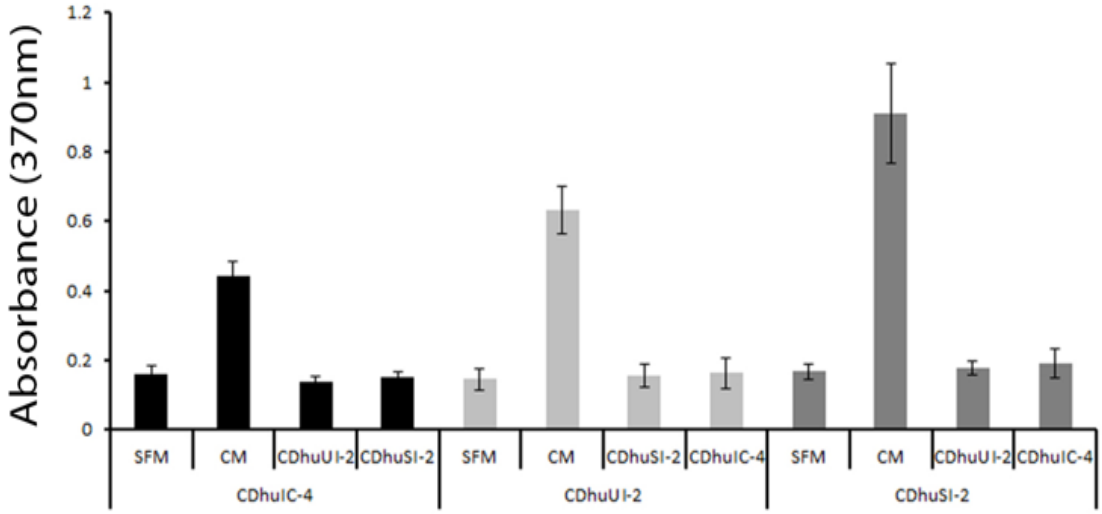


Figure 7. Myofibroblast proliferation is not significantly ($p > 0.05$) altered when cultured in conditioned media.

4.2.3: Myofibroblast Activation

Having observed differences in proliferation between MF subtypes, an α-SMA assay was performed to determine if proliferative changes were matched or associated with changes in metabolic activity.

Alpha-SMA Assay

MF subtype isolated from uninflamed, non-strictured ileum (CDhuUI-2, sample2) showed higher expression of α-SMA than those isolated from both inflamed colon (CDhuIC-4, sample 1) and strictured ileum (CDhuSI-2, sample 3). Expression of α-SMA was lowest in MF from strictured ileum corresponding with the lowest activation state of the three MF groups (Figure 8).

These observations are consistent with a lower metabolically active phenotype of MF behaviour in strictured, but also inflamed bowel.

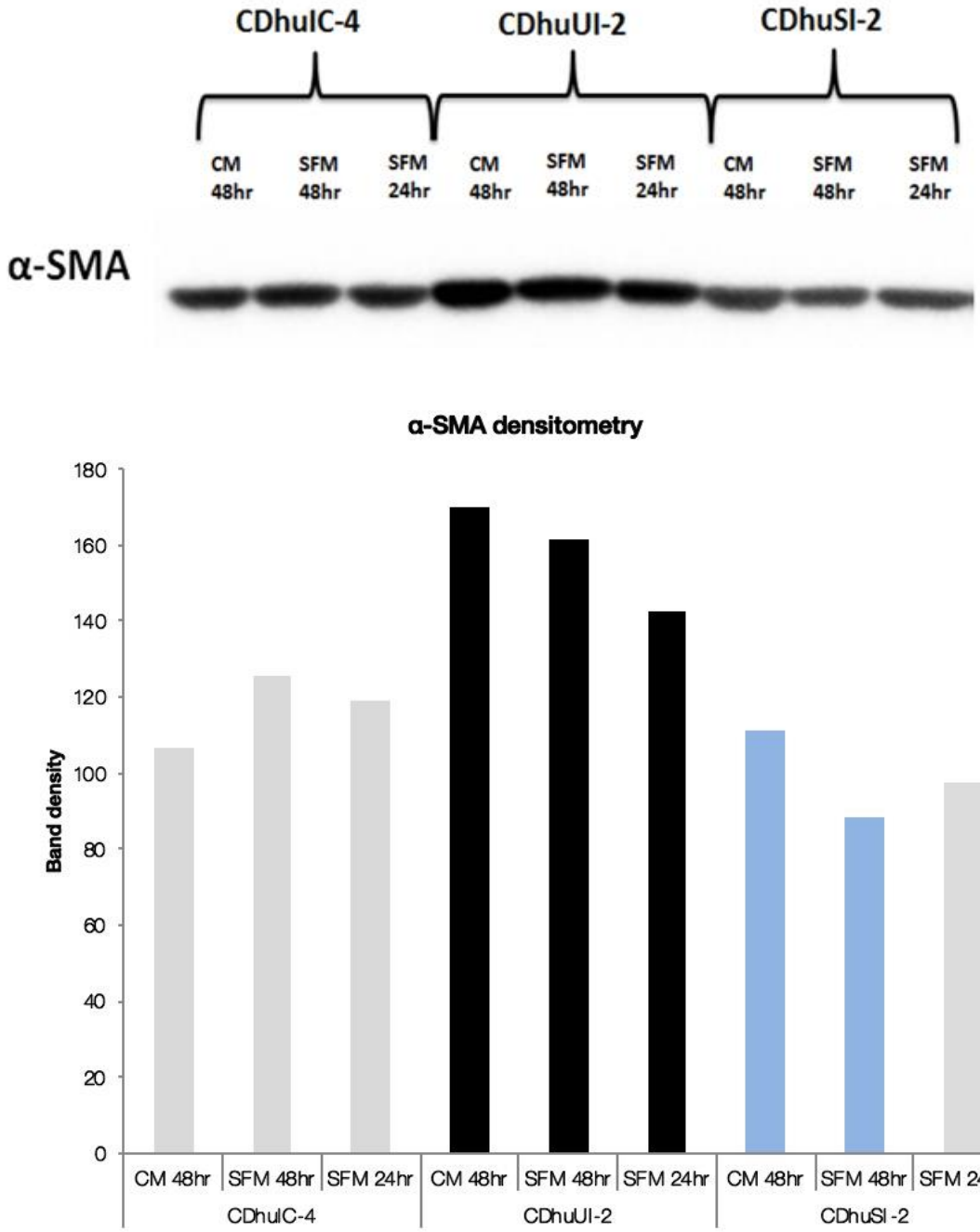


Figure 8. Activation: Intestinal myofibroblasts cultured for 48 hours in complete medium, and 24 hours and 48 hours in serum free medium. CDhuUI-2 MF subtype isolated from uninflamed, non-strictured ileum showed higher expression of α-SMA than those isolated from both inflamed colon (CDhuIC-4) and strictured ileum (CDhuSI-2). Expression of α-SMA was lowest in MF from strictured ileum corresponding with the lowest activation state of the three MF groups. *(Tubulin was used as a loading control for all Western Blots)*

Conditioned Media Incubations (Cross Culture) – Activation

(Note: Colonic MF subtype from patient 1 were not used in the cross-culture activation experiments)

The difference in activation in MF harvested from different localities were further explored for possible influences of the soluble components within the media taken from other completed cell cultures.

Control experiments

In the control experiments, MF subtype isolated from uninflamed, non-strictured (CDhuUI-2) ileum, showed a more activated phenotype than MF subtype from strictured Ileum (CDhuSI-2).

The lower activation state of MF subtype from strictured ileum (CDhuSI-2) compared to that of MF subtypes from uninflamed, non-strictured ileum (CDhuUI-2) was reproduced.

MF subtype from non-strictured ileum incubated in CndM of strictured ileum

Alpha-SMA expression was increased in MF subtype CDhuUI-2 incubated in CDhuSI-2 CndM (Figure 9) and against the control experiments.

The MF subtype from non-strictured ileum remained the subtype with the highest expression of α-SMA.

MF subtype from strictured ileum incubated in CndM of non-strictured ileum

Culture of the MF subtype of CDhuSI-2 (sample 3) in CndM of CDhuUI-2 (sample 2) increased the expression of α-SMA against controls (when compared to CDhuSI-2 culture in both CM and SFM).

Expression of α-SMA was induced to levels similar to that seen in CDhuUI-2. (Figure 9).

ERK Assay

MF subtype from non-strictured ileum incubated in CndM of strictured ileum

In this experiment MF subtype from non-strictured ileum demonstrated an increase in phosphorylated ERK, a downstream protein of activation signalling pathways[165]. (Figure 9).

MF subtype from strictured ileum incubated in CndM of non-strictured ileum

The MF subtype of strictured ileum (CDhuSI-2) demonstrated an increase in phosphorylated ERK. (Figure 9).

These results suggest that there are soluble products present in the culture media from pre-incubated unrelated MF, and paracrine effects may be present.

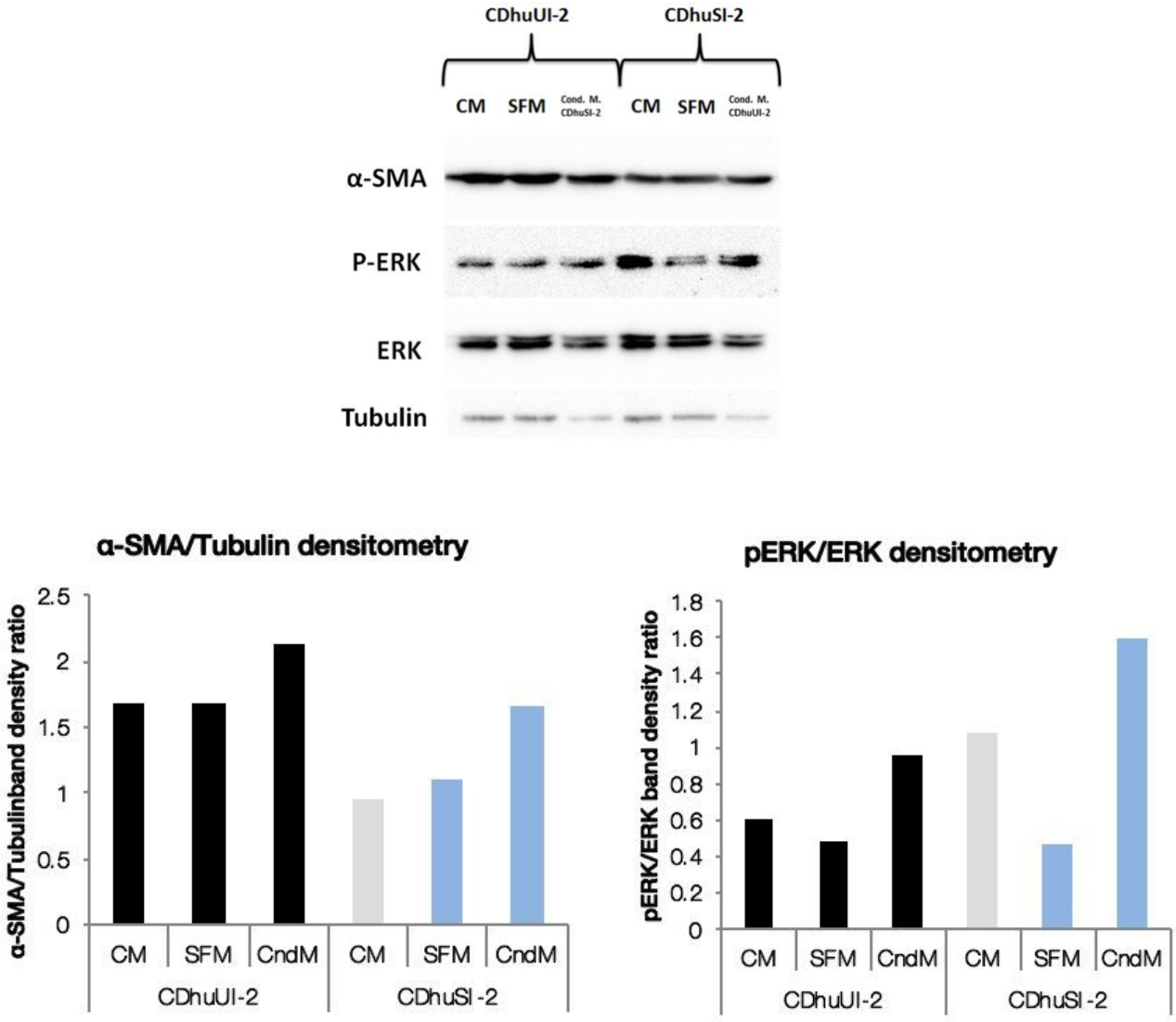


Figure 9. Cross culture – condition media experiments: Activation of myofibroblasts cultured from distinct areas of intestine in an individual Crohn's disease patient. Both myofibroblast sub-phenotypes were cultured in complete medium and serum free medium for 48 hours; in addition, CDhuUI-2 myofibroblasts were cultured in conditioned medium of CDhuSI-2 myofibroblasts, and CDhuSI-2 myofibroblasts in CDhUI-2 conditioned medium, both for 48 hours. CDhuUI-2 myofibroblasts have the highest expression of α-SMA under standard conditions, which is then induced by culture in conditioned medium of strictured MF. CDhuSI-2, whose activation state is lower in standard conditions, was induced by culture in condition medium of non-strictured MF.

TGF-β Activation Experiments with MF Subtypes Incubated with the addition of TGF-β

(Note: MF subtypes from colonic samples were not used in this experiment)

To further investigate external factors of activation, TGF-β, a known profibrotic mediator, was added to each culture medium. MF activation was again assessed using α-SMA and phosphorylation of ERK.

TGF-β concentrations of 2 ng/ml and 10 ng/ml were chosen as previous studies have demonstrated both cell viability and differing activation of MF derived from other organs using these concentrations[153,166,167].

Alpha-SMA Assay

MF subtype from non-strictured ileum

MF subtype from non-strictured ileum (CDhuUI-2, sample 2) demonstrated suppression of α-SMA expression when cultured in medium containing TGF-β1 at both concentrations.

Alpha-SMA was expressed the least in CDhuUI-2 cultured in the 10 ng/ml concentration, reduced to levels similar to those seen in MF subtype of strictured ileum (CDhuSI-2, sample 3) cultured in control conditions of CM and SFM. (Figure 10).

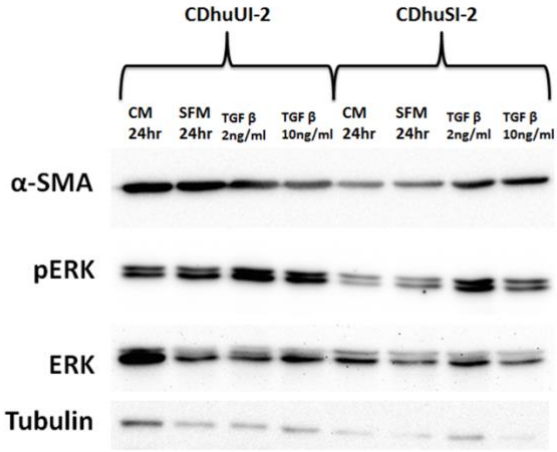
MF subtype from strictured ileum

MF subtype from strictured ileum, CDhuSi-2, demonstrated increased α-SMA expression when incubated, once again in medium containing 10 ng/ml of TGF-β, when compared to controls.

Medium containing TGF-β1 at 2 ng/ml did not produce this effect. (Figure 10).

ERK Assay

Phosphorylation of ERK was uniformly increased in both non-strictured CDhuUI-2 and strictured ileum CDhuSI-2 MF subtypes treated with TGF-β at both concentrations above controls. (Figure 10).



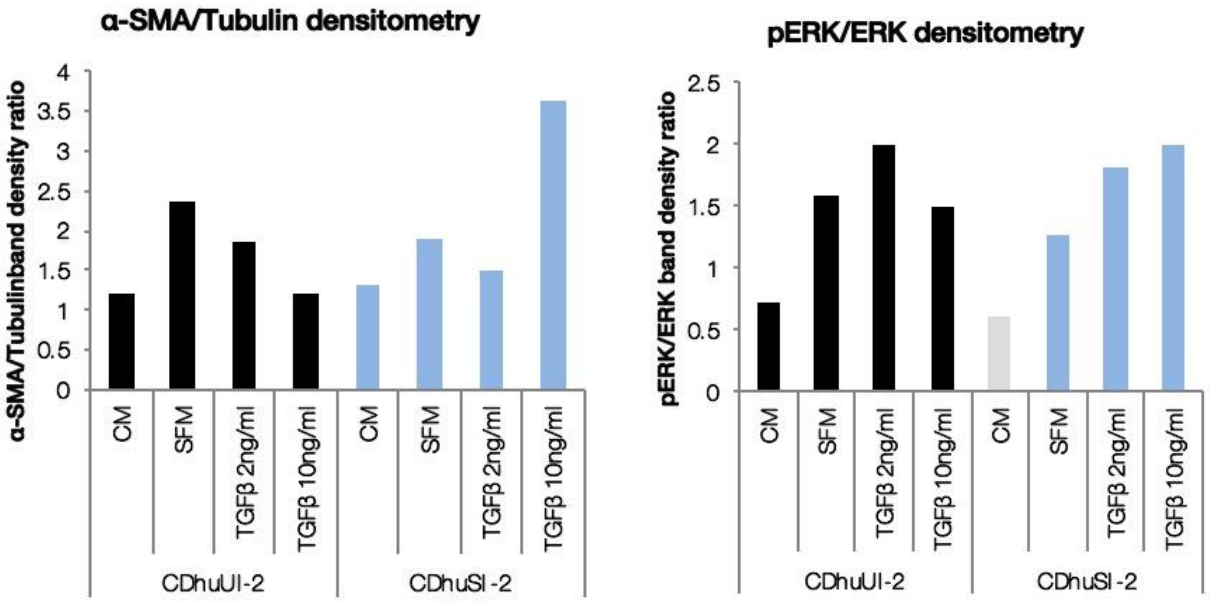


Figure 10. TGF-β1 effect on activation: Activation of myofibroblasts cultured from distinct areas of intestine from an individual Crohn's disease patient. Both myofibroblast sub-phenotypes were cultured in complete medium (CM), serum free medium (SFM) and two concentrations of TGF-β (2ng/ml; 10ng/ml). Activation states in standard culture conditions were reproduced. Treatment with TGF-β 10ng/ml induces activation in CDhuSI-2, as demonstrated by α-SMA, P-ERK and ERK expression. While, TGF-β treatment suppresses α-SMA expression, but increases ERK and pERK expression in CDhuUI-2, with 10ng/ml concentration having the more marked effect.

Collagen 1A Assay

In the control conditions, using CM and SFM, COL1A expression is most pronounced in MF subtype isolated from uninflamed, non-strictured ileum (CDhuUI-2, sample 2). (Figure 11).

Additional treatment with TGF-β at either concentration increased COL1A expression in both MF subtypes from the ileum above controls.

This was most marked in the MF subtype from non-strictured ileum CDhuUI-2 (sample 2) incubation containing 2 ng/ml TGF-β.

In the incubation of MF subtype of strictured ileum, the increase in COL1A was conversely increased when 10 ng/ml was present (Figure 11).

Positive MF activation, including the expression of COL1A, was observed using exogenous TGF-β, but only at a concertation of 10 ng/ml.

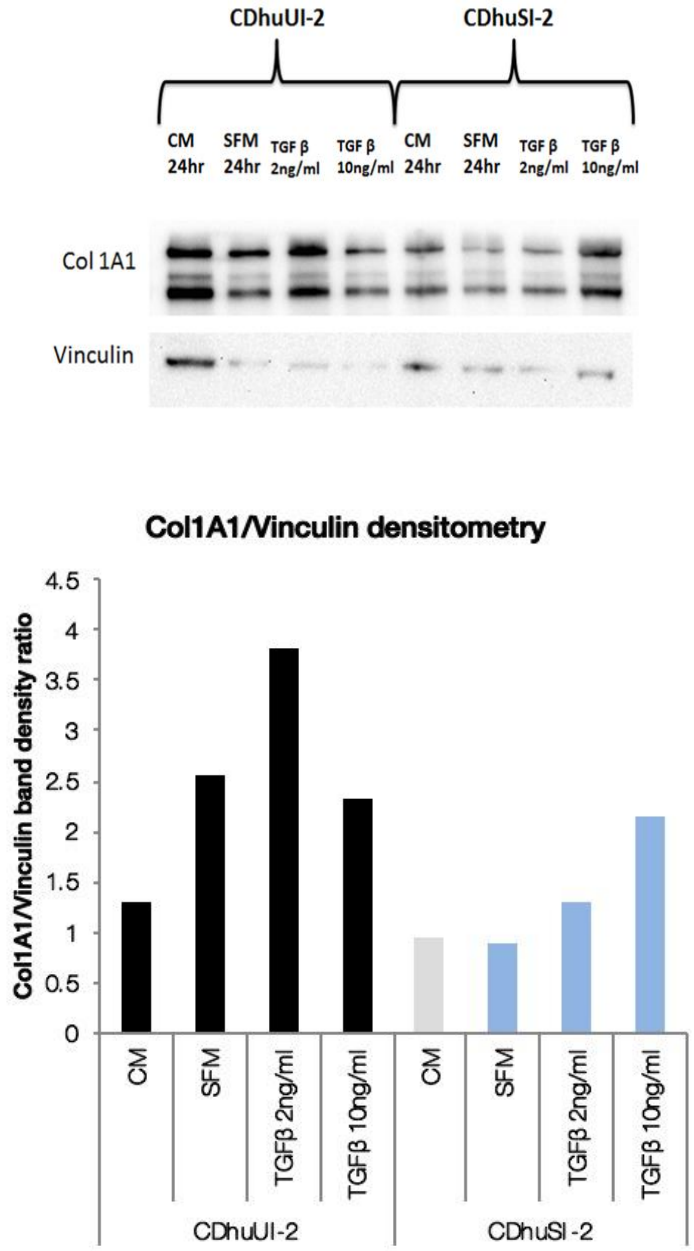


Figure 11. Collagen 1A expression: Myofibroblasts cultured from distinct areas of intestine from an individual Crohn's disease patient. Both myofibroblast subtypes were cultured in complete medium, serum free medium and two concentrations of TGF-β (2ng/ml; 10ng/ml). Activation states in standard culture conditions were reproduced. In control conditions (CM and SFM) collagen 1A expression is most pronounced in CDhuUI-2. TGF-β (dose independent) upregulates collagen 1A expression in both myofibroblast subtypes, most markedly in CDhuUI-2. TGF-β at a concentration of 2ng/ml upregulates collagen 1A expression most in CDhuUI-2, while 10ng/ml TGF-β resulted in the greatest effect in CDhuSI-2.

4.2.4: Wound Scratch Assays

(Note: Colonic cells were not used in this experiment)

Myofibroblast migration is an important and distinct behaviour in wound healing, and may be altered in disease[168].

A wound scratch assay was performed as a physical assessment of MF migration.

- MF subtype from non-strictured ileum (CDhuUI-2) in CM, SFM, CndM of strictured ileum and treatment with 2 ng/ml of TGF-β (Image 4A).
- MF subtype from strictured ileum (CDhuSI-2) in CM, SFM, CndM of non-strictured ileum, treatment with 2 ng/ml of TGF-β (Image 4B).

No significant migration was observed:

MF of both subtypes were seen within each wound scratch in all culture conditions at 24 hours, and more markedly at 48 hours, but appearances are most consistent with the proliferation expected in a culture environment after 24 and 48 hours, rather than migration. (Images 4A and 4B).

Resultant upon the observation of a proliferative rather than migratory response, a proliferative blocking experiment, using mitomycin C, was not performed[169].

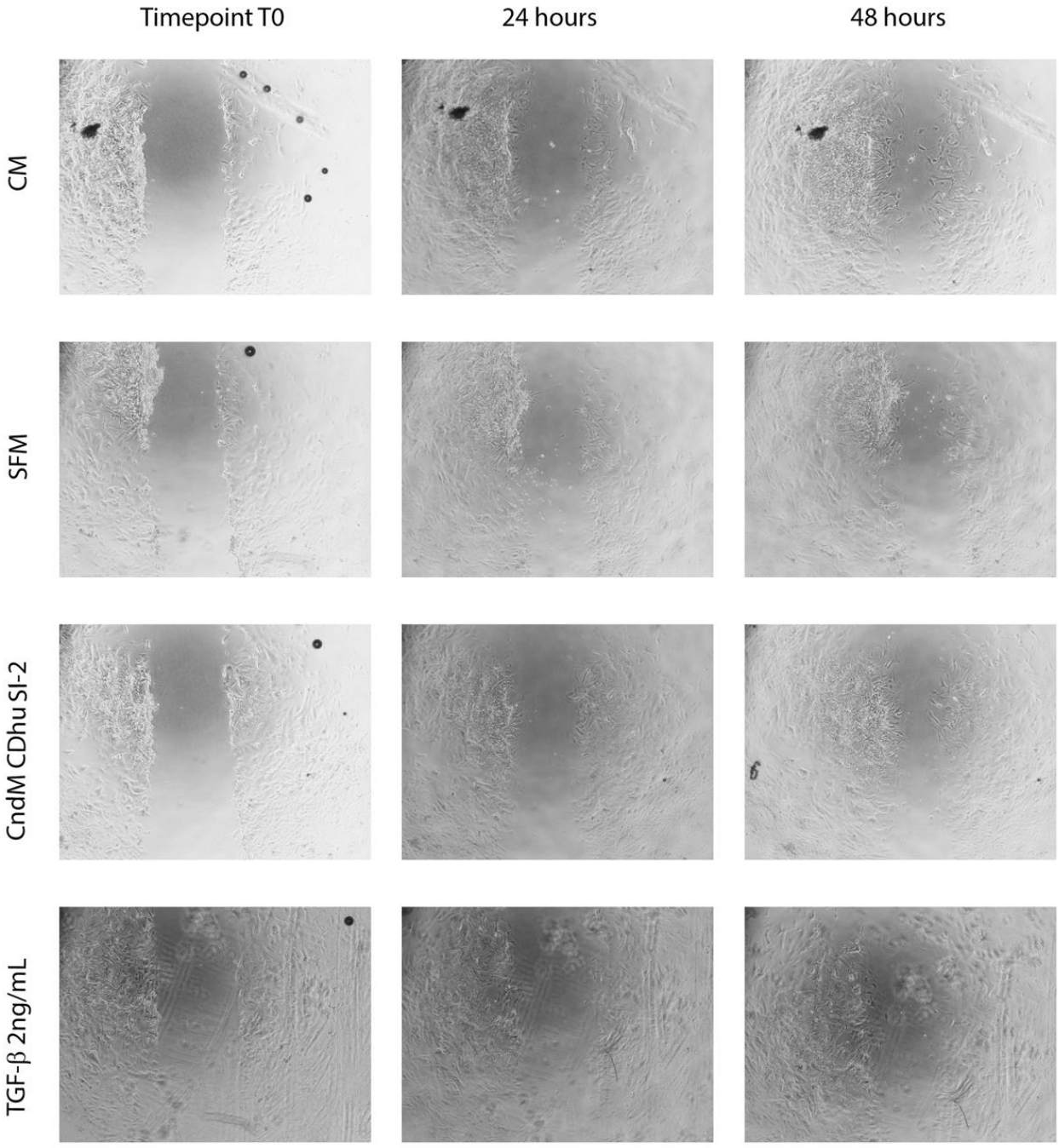


Image 4A. Wound scratch assay of myofibroblasts from non-strictured ileum (CDhuUI-2): Cultured in complete medium, serum free medium, conditioned medium of CDhuSI-2 myofibroblasts and medium treated with 2ng/ml TGF-β. Images taken at time point zero (post scratch), 24 hours and 48 hours. Migration of myofibroblasts is not observed across the wound in any condition. Myofibroblast are seen within the wound at higher time points: The appearance is most consistent with proliferation rather than migration.

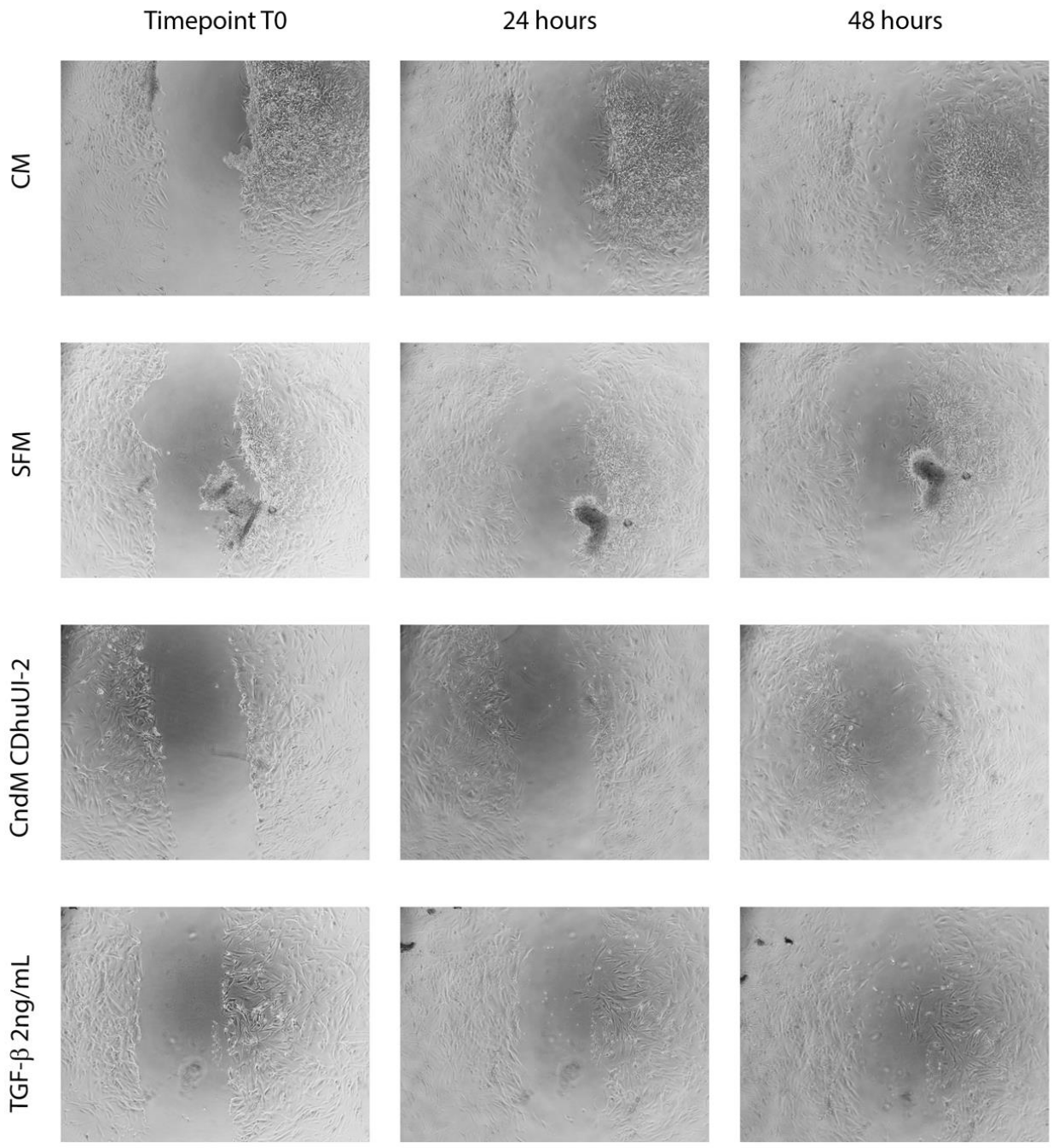


Image 4B. Wound scratch assay of myofibroblasts from strictured ileum (CDhuSI-2): Cultured in complete medium, serum free medium, conditioned medium of CDhuUI-2 myofibroblasts and medium treated with 2ng/ml TGF-β. Images taken at time point zero (post scratch), 24 hours and 48 hours. Migration of myofibroblasts is not observed across the wound in any condition. Myofibroblast are seen within the wound at higher time points: The appearance is most consistent with proliferation rather than migration.

4.3: RNA Analysis Performed with Tissue Samples from Patient 3

In the following set of separate experiments whole tissue samples from an individual CD patient were analysed for activation of known pro-inflammatory and profibrotic genes.

4.3.1: NOD2 and IL-23R

Strictured and non-strictured tissue all demonstrated expression of NOD2 and IL-23R.

Distinct differences in gene expression of NOD2 and IL-23R were observed in the strictured tissue samples from sites 3 and 4.

Within the samples from strictured tissue, site 3 showed reduced expression of both genes, when compared with strictured tissue from site 4, from which an increased expression of IL-23R was demonstrated when compared to all other samples. (Figure 12).

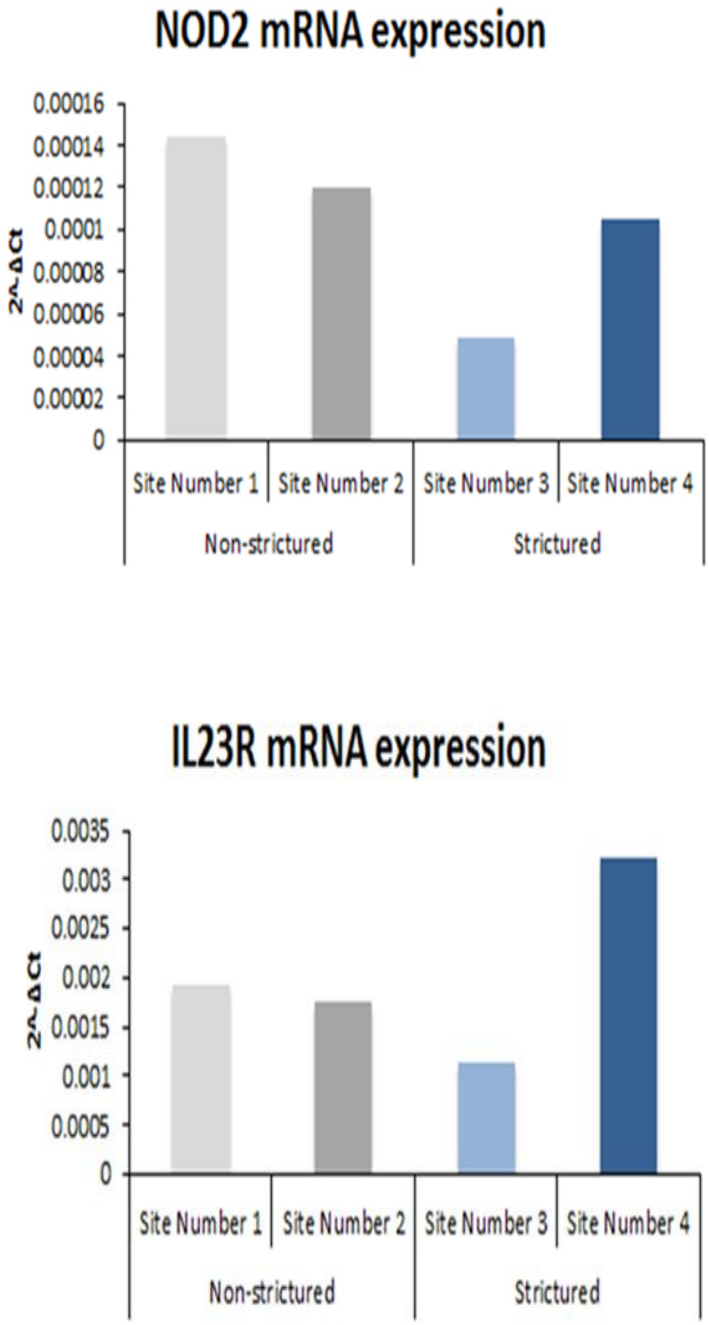


Figure 12. Relative mRNA expression of NOD2 and IL-23R (expressed as 2^-ΔCT) in non-strictured and strictured Crohn's disease tissue samples: Strictured site 3 shows reduced expression of NOD2 and IL-23R, while strictured site 4 increased expression of IL-23R.

4.3.2: Collagen 1A, Collagen 3A, LOX, TNF-α, TGF-β and ACTA2

In strictured tissue from both sites 3 and 4, COL1A, COL3A and ACTA2 gene expression was increased when compared to the samples from sites 1 and 2 from non-strictured sites.

There is little difference in LOX expression in all four samples.

There were similar results obtained for TNF-α and TGF-β except for a reduced expression in the strictured tissue from site 3. (Figure 13).

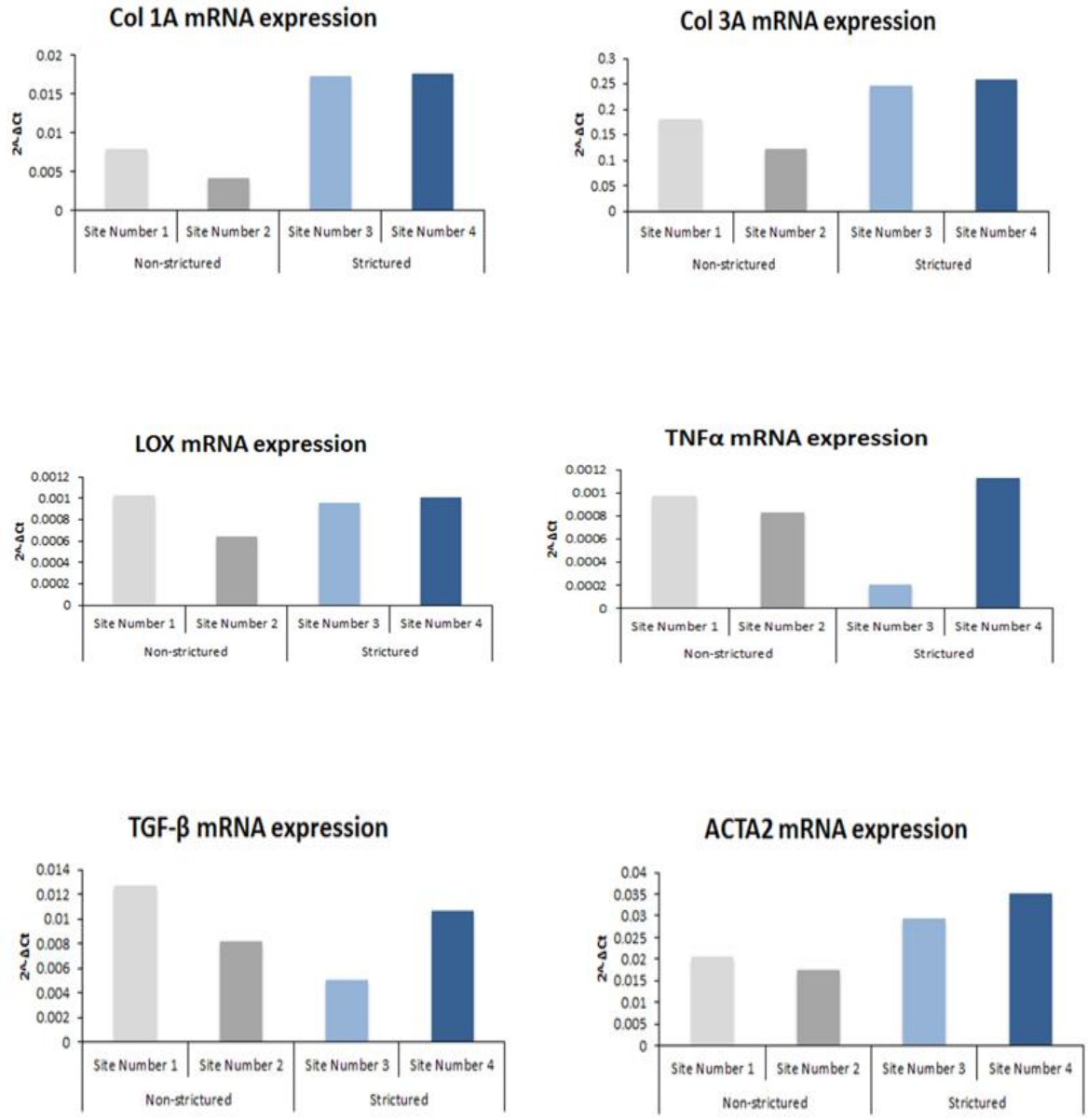


Figure 13. Relative mRNA expression of Collagen 1A, Collagen 3A, LOX, TNF-α, TGF-β and ACTA2 (expressed as 2^-ΔCT) in non-strictured and strictured Crohn's disease tissue samples: Collagen 1A and 3A expression is increased in strictured tissue from sites 3 and 4, compared to non-strictured. LOX expression shows minimal difference between samples. Expression of TNF-α and TGF-β are noticeably reduced in strictured tissue from site 3. ACTA2 expression is increased in strictured tissue from sites 3 and 4, compared to non-strictured.

4.3.3: TIMP-1, MMP-2 and MMP-9

Strictured tissue (sites 3 and 4) showed increased gene expression of TIMP-1 compared to non-strictured tissues (sites 1 and 2).

In contrast, MMP-9 expression was increased in non-strictured tissue (sites 1 and 2) compared to strictured tissue from sites 3 and 4.

There was no noticeable difference in MMP 2 expression in all four tissue sites. (Figure 14).

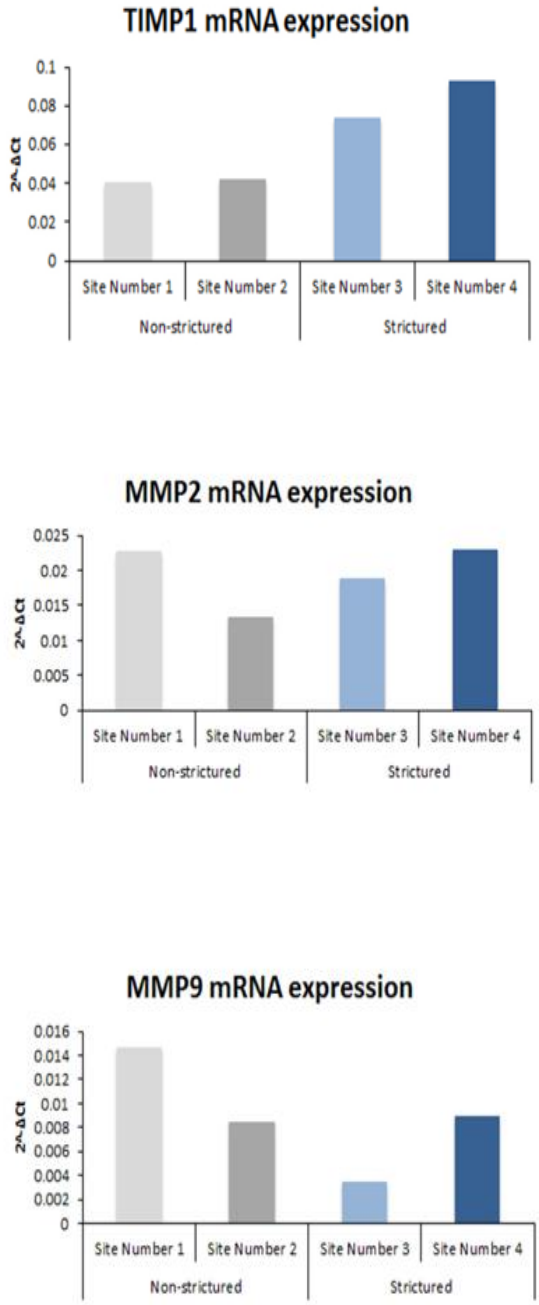


Figure 14. Relative mRNA expression of TIMP-1, MMP-2 and MMP-9 (expressed as 2^-ΔCT) in non-strictured and strictured Crohn's disease tissue samples: TIMP-1 expression is increased in strictured tissue samples from sites 3 and 4, compared to non-strictured; while no clear difference in expression of MMP-2 is seen between samples, expression of MMP-9 is increased in non-strictured tissue samples from sites 1 and 2.

4.3.4: IL-6, IL-1B, IL-8, IL-12B and CCL-2

Overall there was minimal expression of all these marker genes in sites 1, 2 and 3, with the exception IL-6 expression at non-strictured site 2.

Increased expression of all the studied genes was seen in site 4 (strictured tissue).

IL-6 expression was increased in both strictured site 4 and non-strictured site 2. (Figure 15).

These experiments revealed evidence of differences in gene activation of pro-inflammatory and profibrotic genes at different sites.

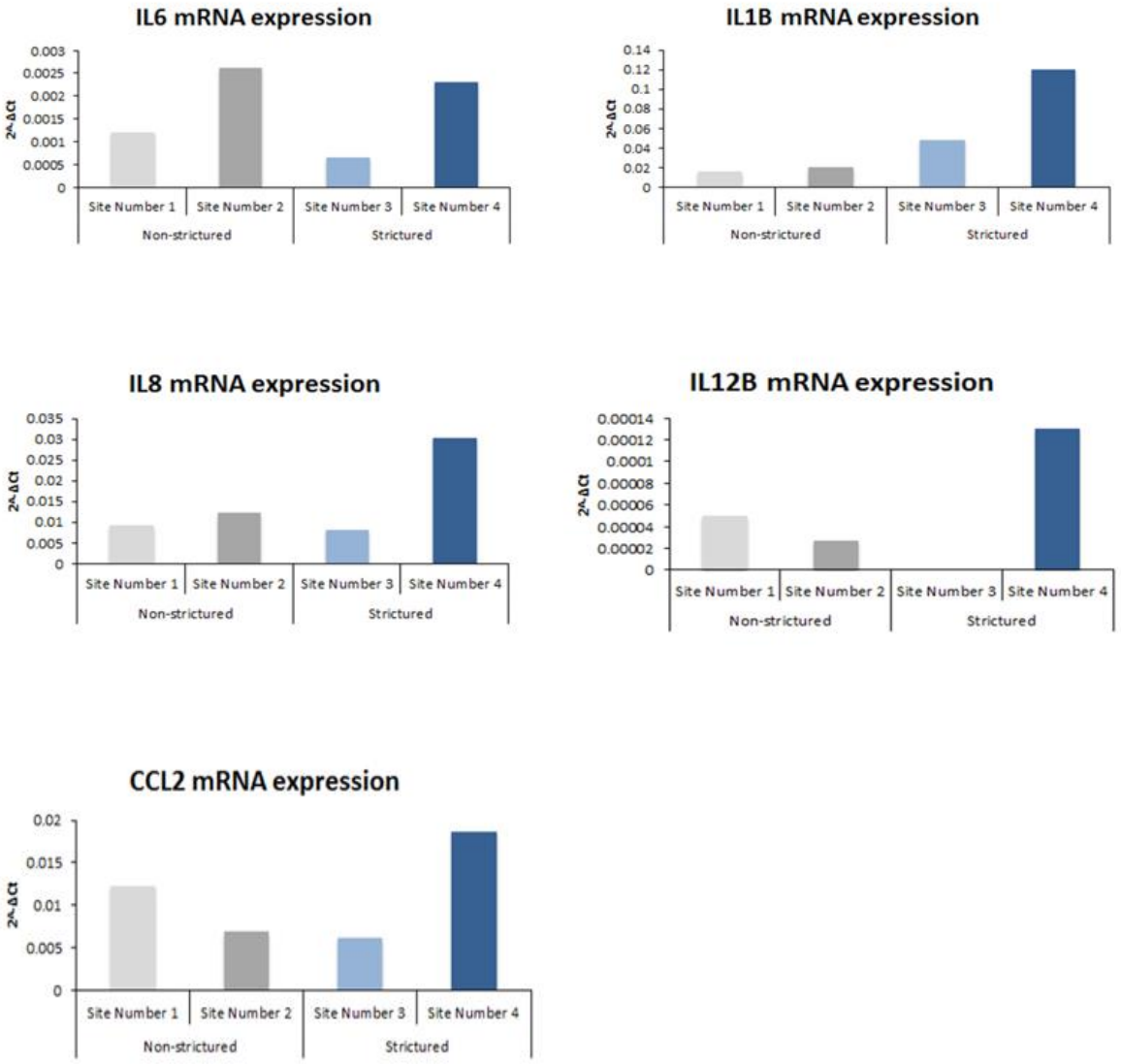


Figure 15. Relative mRNA expression of IL-6, IL-1B, CCL2, IL-8 and IL-12B (expressed as 2^-ΔCT) in non-strictured and strictured Crohn's disease tissue samples: IL6 expression varies between sites, whether strictured or not: Similar levels of increased expression is seen in non-strictured site 2 and strictured site 4, while both non-strictured site 1 and strictured site 3 have comparatively reduced expression. IL-1B expression is increased in strictured tissue, particularly from site 4, compared to non-strictured. CCL2 expression is most increased in strictured tissue from site 4. There is increased expression of IL-8 in tissue from strictured site 4, compared to the other three sites, which have similar levels of low expression. IL-12B expression is seen most increased in strictured tissue from site 4, although was not identified at all in strictured site 3; low expression is seen in non-strictured tissue.

5: DISCUSSION

5.1: Overview

Crohn's Disease (CD) is an elaborate immune mediated inflammatory disease, with development and progression influenced by many factors, including environmental, genetic, and the host microbiome[4].

Extensive research continues but currently has been unable to define a pathogenic pathway which culminates in the clinical picture of CD[170].

Precise inflammatory triggers remain unknown and particularly those that determine the ultimate clinical phenotype[171].

In particular, the percentage of CD patients that develop fibrotic disease poses one of the major clinical challenges[31].

it is not even possible to identify those who will develop the disease, even in monozygotic twins the concordance rate is only 20 to 50%[172].

The identification of predictive disease markers would ultimately influence the management and treatment protocols, but at present this remains elusive[173].

Similarly, within the CD spectrum, identification (either clinically, genetically or by cellular technology), of the triggers of the fibrotic pathway might permit interruption of this pathological process[174].

Intestinal fibrosis is a highly complex process, and the precise aetiology remains unknown[81].

Transmural inflammation in CD cannot be simplistically regarded as a 'wound', or the reparation response of this insult as healing. In fact, inflammation itself is an unpredictable variable in the fibrogenesis of CD and furthermore the fibrotic process can continue in the absence of inflammation[31,97].

The investigation of profibrotic pathways in CD becomes very pertinent to providing any prospect of a therapeutic intervention[94].

5.2: This Study

The approach of this study is to compare the behaviour of the major profibrotic cell, the MF, in tissue samples taken from CD patients in different sites of the GIT. In addition, the behaviour of cells within, distant from, and adjacent to, strictured inflammatory tissue has revealed promising differences of cellular behaviour. Changes in these areas are potentially driven by paracrine mechanisms.

The isolation of myofibroblasts (MF) from human tissue of patients has provided a realistic platform from which to study the behaviour of these cells *in vitro*.

Although these MF have been removed from their biological environment and subsequently cultured, they have all been subjected to the same experimental conditions and comparisons of their behaviour can reasonably be made.

Further advances in maintaining a sample of original cells through several passages of culture cycles means that there is sufficient biological material for experimentation. Isolated MF can withstand freezing (and transportation) to the UK.

The laboratory use of human cells (rather than those from other animal species), has the advantage of immediate human relevance, but all experiments performed illustrate the precarious nature of working with human cells that are not immortal (unlike, for example, HeLa cell lines[175]).

This point is illustrated by the encouraging success of some cell samples from ileal tissue, but the earlier mortality of a line of colon derived cells, which limited their use in some experiments.

In this study both the morphology and the synthetic ability of revived MF, which are cultured on through several passages, is demonstrated.

Research in CD fibrosis using primary human MF frequently focuses on cells isolated from intestinal tissue from CD patients, not limited to a fibrostenotic phenotype, or strictured tissue alone, and comparisons made with MF isolated from separate CD patients and/or healthy controls[95].

It is demonstrated, perhaps unsurprisingly, that MF from each of these patient groups have distinctly differing phenotypes.

One unique feature at the outset of this research project was to investigate whether and how MF derived from strictured and non-strictured intestine in an individual CD patient would present different features and also influence one another.

Two MF subtypes, CDhuSI-2 and CDhuUI-2, were isolated from an individual patient: CDhuSI-2 MF from strictured ileal mucosa, and CDhuUI-2 MF from adjacent uninflamed, non-strictured ileal mucosa.

Following this preliminary work other investigators have used MF isolated from multiple locations in an individual with a fibrosing phenotype[176–178].

5.3: Myofibroblast Behaviour

In this study the morphological features of the isolated and revived MF demonstrated appearances consistent with normal live cells.

Proliferation was quantified using a nucleoside-analogue incorporation assay, in this case utilising the thymidine analogue, BrdU. The technique is considered appropriate for assessing in vitro cell proliferation over time as BrdU is stably

incorporated into DNA and preferred in our laboratory as there is no handling of radioactive material and the required equipment was available and accessible. This is an end point assay as staining requires cell membrane permeabilisation and fixation[179].

Although this technique cannot identify cells that have undergone numerous rounds of division, this limitation was not considered relevant for the purposes of this study[179].

The other most commonly recognised general approaches to assess cell proliferation, cell cycle associated protein assays and the use of cytoplasmic proliferation dye, could be considered for future validation experiments, although no single method is without both advantages and disadvantages[179].

In the complete culture medium (CM) the MF demonstrated both proliferation (maintained for several passages), and synthetic activity which were both attenuated, but still present when the serum component was absent (SFM).

These baseline incubations, having no other interventions, were used as comparative controls in the experiments involving modification of the culture parameters.

MF culture provided reliable passaging and all the ileal MF subtypes were studied most comprehensively.

Myofibroblast proliferation was best observed when cultured in the optimal CM. MF isolated from quiescent strictured ileum had increased proliferation, not only when compared to MF from uninflamed non-strictured ileum but also when compared with inflamed CD colon.

This observation does concur with an expectation that MF from within a stricture would be biologically active in the fibrotic process[31,95].

Subtypes of MF originating from inflamed colon did not show increasing proliferation. Furthermore, MF originating from macroscopically normal ileal tissue adjacent to the stricture did proliferate more than MF originating from inflamed tissue, observations that might be consistent with MF priming rather than prior exposure to inflammatory activity.

Despite the magnitude of the proliferation of MF from the stricture, markers of synthetic activity were the least expressed, an observation that would be consistent with this population of cells not driving the local fibrosis.

The markers of MF activity were greatest in MF isolated from adjacent uninflamed, non-strictured ileum, and if were a consistent finding would strongly indicate paracrine signalling.

Such mechanisms have already been described for other fibrogenic models[146–149].

The incubation experiments with the known fibrogenic mediator TGF-β which has autocrine and paracrine effects, suggest an active role of this mediator[81,97,180].

Treatment with TGF-β induced activation of MF isolated from strictured ileum, demonstrated by increased α-SMA expression and up-regulation of phosphorylation of ERK, suggesting that this population of cells are capable of induced synthetic activity.

In the synthetically active MF subtype from non-strictured ileal tissue, TGF-β further increased expression of only one marker (ERK phosphorylation) but suppressed rather than enhancing α-SMA expression.

This reduction of an important profibrotic mediator could be anomalous but might be indicative of feedback control and further evidence of paracrine mechanisms.

Further, the intracellular expression of COL1A, a major structural protein of the ECM produced in abnormal excess in CD fibrosis[81,89], was investigated as MF are the known predominant source[181].

Consistent with the increased synthetic activity of MF from non-strictured ileum, COL1A expression was most pronounced in this MF subtype.

However, and importantly, TGF-β stimulated the previously less active MF cells from strictured ileum and further enhanced the others.

5.3.1: Cross Incubation Experiment

These experiments were designed to observe any influence of the medium taken from one set of cultured MF subtype on the behaviour of another MF subtype as we have previously described[146–149].

The identification of any change in the newly exposed MF to the culture medium taken from another subtype, would add support to the presence of incubated media accumulating active soluble products capable of signalling another cell[151].

The results were consistent with the exchanged media containing signalling products that in all combinations, although failing to further promote proliferation, did produce positive changes in synthetic expression.

Actively synthetic MF subtype from non-strictured ileum, stimulated synthetic activity in those MF that had previously demonstrated less activity. This evidence of signalling from soluble media was further consolidated by the observation of MF subtype, even with less synthetic activity at baseline, once again being capable of stimulating their more initially active counterpart.

Paracrine effects are likely to be present influencing MF activity, but their absence in the proliferation experiments, might indicate autocrine mechanisms in this process and needs further investigation.

5.3.2: Myofibroblast mobility

The physical behaviour of MF in fibrogenesis is also important[182].

It has been previously demonstrated that MF isolated from patients with penetrating CD have faster rates of MF mobility in wound assays, than those isolated from patients with stricturing disease[104,105,183].

Data from this study failed to demonstrate migration either by untreated cells or those treated with TGF-β. Proliferation was seen, confirming cell viability, but ones which were devoid of migratory ability.

In this 2-dimensional model, had the wound been observed to close completely at either 24 or 48 hours, it would be important to determine any contribution of MF proliferation to ensure a clear assessment of migratory activity[169].

Therefore, in a separate set of experiments, MF proliferation would be blocked by the addition of mitomycin C into the experiment[169].

Subsequent observations of any wound closure could then be attributed to MF migration.

5.4: RNA analysis

A single study has previously observed differences in strictured and non-strictured mucosa in individual CD patients, reporting profibrotic increased TGF-β, collagen and TIMP-1, but decreased antifibrotic MMP-3 and -12 protein expression in mucosa of strictured intestine[155].

In this study, colonic samples taken from across an uninflamed stricture and adjacent normal mucosa provided tissue to investigate the expression of gene markers of fibrogenesis.

It is important to note that any disparity between identified gene expression and gene product does not necessarily mean that the observations are erroneous. RNA analysis was performed on whole tissue rather than MF cells, and therefore included all the cellular constituents, and activity of other possible downstream factors, for example protein enzymatic degradation, might produce disparity between expression and end protein biological activity.

An example, from this study, is the ACTA2 gene expression, encoding α-SMA, was found greatest in strictured tissue. This observation contrasts to the activity experiments of isolated MF from strictured tissue which showed less α-SMA expression than non-strictured samples.

The results from RNA experiments clearly indicate that there is a balanced interaction of the genes involved in the fibrotic pathway.

Supporting fibrosis, gene expression of COL1A and COL3A, both major structural protein components of the ECM[81,89], was greatest in tissue obtained from the stricture where the greatest accumulation of ECM is found. The expression of TIMP-1, a profibrotic ECM regulator[180], is also present in the fibrotic stricture.

Expression of MMP-9, involved in ECM degradation, and whose expression is suppressed by TIMPs[29,89], was higher in non-strictured tissue where TIMP expression was less.

Non-strictured tissue gene expression revealed increases in the key profibrotic mediator TGF-β1, in contrast.

In the same tissue, increased expression of TNF-α, IL-6 and CCL2, all mediators known to exert a profibrotic influence on the TGF-β fibrosis pathway[58,184], was also observed.

Within the concept of a balanced process driving fibrosis, expression of IL-12B and IL-8, cytokines both inhibitors of the TGF-β fibrotic pathway[58,73], was complementarily low in this tissue.

Contrary to a reasonable presumption that non-strictured tissue itself would not be promoting fibrosis, it is tempting to consider whether this activity of non-

strictured tissue is reflecting a process permitting remote biological influence on the adjacent strictured area.

5.5: Summary

The isolated cells can be stored and revived and remain viable such that they proliferate, retain normal morphological features and respond to experimental conditions that can be followed objectively.

The experiments indicate that MF exert two important modalities of behaviour, that of cellular proliferation and of synthetic activity, and which differs between the different sites of CD involvement.

There is evidence that the synthetic activity of any particular subgroup of MF, determined by the intestinal site from which they were harvested, can exhibit likely paracrine properties, which influence the synthetic behaviour of others.

In contrast, it seems more likely that proliferative response of MF subtypes relies on local autocrine determinants.

5.6: Conclusion

Experimental observations made in this study do add support to a clinical impression that inflammation is not the primary driver of Crohn's fibrosis[31,95,97]. This local and remote influence is also reflected in gene expression in CD tissue. The findings do suggest that a balance of profibrotic and antifibrotic mechanisms are operating[185], and with a greater sample size from a large patient group, would provide tissue that could explore the validity of this concept.

Confirmation of these early observations would provide new prominence to what would otherwise be considered benign intestinal mucosa adjacent to a stricture, intimating that its constituents, particularly MF, through paracrine signalling, significantly contribute to the development and progression of neighbourhood fibrosis.

6: FUTURE STUDIES

1: Identifying fibrotic 'initiators' and 'drivers' of the fibrostenotic phenotype in individual patients

Identifying patients with a stricturing phenotype is an important clinical milestone in the management of their disease. Such patients may exhibit an identifiable and reproducible pattern of gene expression[186,187].

Such data could then be used in longitudinal studies of all CD patients potentially predetermining their likelihood of developing stricturing disease[186,187].

A. Biopsies will be taken from strictured and non-strictured areas of each patient, RNA extracted, and qPCR performed.

In this way a network and pathway analysis can be performed with the selected list of specific genes[185]. The combination of employing STRING (Search Tool for the Retrieval of Interacting Genes/Proteins), a database of known and predicted protein interactions (http://string-db.org/), with the Database for Annotation, Visualisation and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) software can reveal a complex network of genes/proteins/pathways interactions involved in the process of fibrostenosis during CD[188].

B. Alterations in protein/gene expression will be further analysed[185]: MF from a CD patient with stricturing phenotype (i.e. strictured MF versus non-strictured MF), and ideally against CD samples who lack a fibrotic phenotype.

The *in vitro* findings will be explored in human biopsies to further confirm the importance of these alterations during CD fibrogenesis[185].

2: Establishment of successful myofibroblast isolation protocol in this laboratory

Surgically resected tissue and biopsy specimens will be used to refine and establish a local MF isolation protocol and maintain the cells in a viable state.

Recent advances in the availability and practicality in assessing gene expression in tissue samples will facilitate further studies at an individual cellular level.

Primary cell interrogation still has relevance, providing a faithful transcriptomic and proteomic profile and closer proximity to physiological function and response[189].

Unfortunately the requirement of cell culture for specific nutrient substrates, and the acquisition of a senescent phenotype, resulting in irreversible cell cycle arrest, limits the amount of material that can be obtained, preventing their use

in large-scale disease and therapy modelling[190], but targeted mechanistic studies would be possible and relevant.

3: Reproduction of experiments with multiple, multi-patient samples

A larger cohort of patients will provide the variety of diseased tissue samples required not only to increase the yield of experimental tissue but also to validate observations made by multiple repetitions of the described experiments on both intestinal tissue and human MF obtained from strictured and non-strictured areas from individual CD patients.

This will require a stable and larger population of MF subtypes than achieved so far.

In vitro MF behaviour, paralleled with RNA gene expression studies would be the initial experiments most likely to produce useful data[186,187,189].

APPENDICES

Appendix 1: Further Acknowledgments

Above all, I thank my family.

To my parents, Mary and Hugh, who created a home where to work hard and do your best was put above all other chores. A life devoid of dishwashing but full of encouragement instilled intrigue and a work ethic required to pursue a career in medicine.

Without the love and companionship of my wife, Carla, not only may I have never started, but almost certainly never finished, although I am confident submission will come as a blessed relief.

To my daughters, Molly and Ruby, who did not ask for their father to have a separate life of research and clinical work, both being born ‘in the middle of it all’. Their presence generates a father’s desire to strive for the highest of standards, cultivating a healthy fear of failure, without which this thesis could not have been completed.

Appendix 2: Ethics favourable opinion

NRES Committee London - Riverside
Bristol Research Ethics Committee Centre
Level 3 Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Telephone: 0117 342 1385

02 December 2014

Professor Massimo Pinzani
UCL Institute of Liver and Digestive Health
University College London Royal Free Campus U3
Rowland Hill Street, London
NW32PF

Dear Professor Pinzani

Study title:	**Exploring the Mechanisms of Intestinal Fibrosis in Stenotic Phenotypes of Crohn's Disease**
REC reference:	**14/LO/1701**
Protocol number:	**14/0244**
IRAS project ID:	**144029**

Thank you for your letter of 06 November 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Assistant, Miss Maeve Groot Bluemink, nrescommittee.london-riverside@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering letter on headed paper [Ethics Cover Letter 28_11_2014]		28 November 2014
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance Certificate]	1.0	14 July 2014
IRAS Checklist XML [Checklist_08092014]		08 September 2014
IRAS Checklist XML [Checklist_06112014]		06 November 2014
IRAS Checklist XML [Checklist_11112014]		11 November 2014
Participant information sheet (PIS) [PIS Version 1.2 30th October 2014 (Pts without CD) with changes highlighted]	1.2	30 October 2014
Participant information sheet (PIS) [PIS Version 1.3 2nd December 2014 (Pts with CD) with changes highlighted]	1.3	02 December 2014
REC Application Form [REC_Form_11112014]		11 November 2014
Research protocol or project proposal [Protocol]	1.0	29 July 2014
Response to Request for Further Information [Email from applicant with response to PO]		06 November 2014
Summary CV for Chief Investigator (CI) [Professor Massimo Pinzani Summary CV]	1.0	25 June 2013
Summary CV for student [Dr Thomas Shepherd Summary CV]	1.0	04 August 2014
Summary CV for supervisor (student research) [Dr Charles Murray Summary CV]	1.0	09 September 2013

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

14/LO/1701	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely

pp
Dr Sabita Uthaya
Chair

Email:nrescommittee.london-riverside@nhs.net

Enclosures: "After ethical review – guidance for researchers" *[SL-AR2]*

Copy to: *Ms Suzanne Emerton, University College London Hospitals NHS Foundation Trust*
Mrs Liba Stones, Acting R & D Manager Royal Free Hospital NHS Foundation Trust

Appendix 3: Ethics favourable opinion amendment

NHS
Health Research Authority
London - Riverside Research Ethics Committee
Level 3 Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Tel: 0117 342 1385

05 February 2016

Dr Thomas Shepherd
Clinical Research Fellow
Institute for Liver and Digestive Health
UCL, Royal Free Campus
Pond Street

Dear Dr Shepherd,

Study title:	**Exploring the Mechanisms of Intestinal Fibrosis in Stenotic Phenotypes of Crohn's Disease**
REC reference:	**14/LO/1701**
Protocol number:	**14/0244**
Amendment number:	**Modified Amendment to SA1**
Amendment date:	**02 February 2016**
IRAS project ID:	**144029**

Thank you for submitting the above amendment, which was received on 02 February 2016. It is noted that this is a modification of an amendment previously rejected by the Committee (our letter of 27th January 2016 refers).

The modified amendment has been considered on behalf of the Committee by the Chair.

Ethical opinion

I am pleased to confirm that the Committee has given a favourable ethical opinion of the modified amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved are:

Document	*Version*	*Date*
Notice of Modified Amendment [AmendmentForm_ReadyForSubmission]	Modified to SA1	01 February 2016
Participant consent form [Consent (Pt with CD) V1.5 Feb 16 - clean]	1.5	01 February 2016
Participant consent form [Consent (Pt with CD) V1.5 Feb 16 - tracked]	1.5	01 February 2016
Participant consent form [Consent (Pt without CD) V2.2 Feb 16 - tracked]	2.2	01 February 2016
Participant consent form [Consent (Pt without CD) V2.2 Feb 16 -	2.2	01 February 2016

clean]		
Participant information sheet (PIS) [PIS (pt with CD) V1.4 Oct 2015]	1.4	30 October 2015
Participant information sheet (PIS) [PIS (pt without CD) V1.3 Oct 2015]	1.3	30 October 2015
Participant information sheet (PIS) [Tracked changes PIS - pts without crohns V1.3 Oct 2015]	1.3	30 October 2015
Participant information sheet (PIS) [Tracked changes PIS pts with crohnsV1.4 Oct 2015]	1.4	30 October 2015
Research protocol or project proposal [PhD Protocol (Version 1.2 29th December 2015)]	1.2	29 December 2015
Research protocol or project proposal [PhD Protocol Version 1.2 29th December 2015 (changes highlighted)]	1.2	29 December 2015

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/LO/1701:	**Please quote this number on all correspondence**

Yours sincerely

Pp [redacted]

Dr Margaret Jones
Chair

E-mail: nrescommittee.london-riverside@nhs.net

Copy to: *Liba Stones, Royal Free London NHS Foundation Trust*

Appendix 4: Participant information sheet for patients with Crohn's disease

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

Participant Information Sheet for Patients with Crohn's Disease

---

Mechanisms of Intestinal Fibrosis in Stenotic Phenotypes of Crohn's Disease
(Student Study)

INTRODUCTION

Royal Free London NHS Foundation Trust ('the Trust') is a leading teaching hospital and bio-medical centre, where many tissue and cell samples are taken from patients each day for laboratory tests. Examples of these specimens include blood samples, tissues removed during operations, and urine.

The Trust is a major centre for research into the causes of diseases and their treatment. For detailed information about our research, please visit our website: www.royalfree.nhs.uk

Invitation

You have been invited to take part in this study of Crohn's disease by donating gut tissue specimens and peripheral blood.

We are inviting you to take part as you have Crohn's disease and require either an endoscopy or surgery as part of your normal care.

Introduction to this research project

Crohn's disease (CD), a type of inflammatory bowel disease, causes inflammation along the entire digestive tract leading to disabling symptoms such as abdominal pain and bloody diarrhoea. The exact cause is unknown, but there is an abnormal response by the body's defences – immune system – and it attacks itself. Worldwide it affects anywhere between 5 to 10 people per 100,000 per year.

While there is no cure, modern medications that suppress the immune system have greatly improved patients' symptoms and reduced the need for surgical removal of diseased bowel by reducing inflammation. However, a proportion of patients will develop areas of narrowing in their bowel – strictures – that cause blockages. Modern medications do not treat strictures and patients often require multiple operations to remove them. Little is known about strictures in CD and why they develop.

Our research will focus on strictures to better understand how and why they develop, with the ultimate goal of proposing different and new treatments. Specifically, we will use strictured areas of bowel that have had to be surgically removed from patients with CD in addition to samples of their blood to investigate at a microscopic level the mechanisms involved in stricture development.

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

WHATS INVOLVED?

What is the purpose of this specific study?

Using samples of gut and blood we aim to research causes of gut narrowing (fibrosis) in inflammatory bowel disease, specifically Crohn's Disease, at a microscopic level.

Why have I been chosen to donate tissue and blood?

We are inviting you to take part as you have Crohn's disease:

All patients with Crohn's disease requiring endoscopic (colonoscopy or flexible sigmoidoscopy) evaluation of their disease and/or surgical resection of a narrowed (strictured) section of Crohn's affected bowel are being invited to donate biopsied or resected tissue and a blood sample.

What would taking part involve?

You have been invited to take part as either endoscopic investigation and/or surgery has been decided to be the most appropriate investigation or treatment as part of normal care.

As the study is laboratory based your direct involvement is limited, and as such, additional time, outside that required for normal standard of care, is small.

Pre-procedure consultation will allow you to ask any questions prior to informed consent being obtained.

Peripheral blood and tissue samples will be obtained at the time of your endoscopy or surgery for the purpose of our research study. A maximum of 10 millilitres of blood and 8 biopsies (if you are having an endoscopy) will be taken in addition to what would be required as part of standard care. This number of biopsies will add approximately an extra 4 minutes on to the procedure time.

If you are having surgery a piece of removed tissue will be reserved for experiments.

Subsequently, the investigator will not require direct patient contact.

Possible benefits

The nature of this research is such that immediate benefit to a specific participant is not anticipated. However, in time we hope results will contribute to benefit novel management in inflammatory bowel disease patients as a whole community.

We hope to further knowledge in Crohn's Disease that we would actively disseminate in an understandable format so patients, and the lay public at large, better understand the process of scarring with stricture formation and rationale for current treatment and the possibility of developing new therapies.

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

Possible disadvantages and risks

Additional burdens for research participants will be limited to time. You are required to spend non-pressurised time with this information leaflet, which will precede a consultation to answer potential questions and obtain informed consent. We will aim to limit additional visits to the hospital by incorporating this in to routine required hospital visits, although additional consultation time (above that of a normal outpatient visit) will be required.

Otherwise, adverse events will be associated with the standard of care in patients having endoscopic assessment of their Crohn's disease and/or surgical resection of bowel, and these will not be enhanced by study participation.

The operating surgeon will discuss risks involved with the surgery with you.

The doctor performing your endoscopy will discus risks involved with your procedure. A flexible sigmoidoscopy with biopsies has a 1:15,000 chance of perforation (making a tear in the gut), while a colonoscopy with biopsies has a 1:1000 chance of perforation.

Consent

Consent will be obtained only by medically qualified members of the research team.

Patients identified as suitable candidates for donation, use and storage of human tissue and peripheral blood will be appropriately consented.

The independent consent form and discussion will be located in the patients source document (paper patient notes are in use at The Royal Free Hospital).

For non-English speaking participants the Royal Free Hospital has a telephone translation service in place and written information will be translated.

Every effort will be made to accommodate individual patients needs.

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

SUPPORTING INFORMATION

What will happen to the samples I give?

Donated tissue and blood samples will initially be frozen and stored. Subsequently they will be used in experiments for this research study. The experiments will take place in the medical laboratories located at The Royal Free Hospital.

What happens to your tissue after it has been used?

Samples of blood are only kept for a few weeks and are then disposed of by incineration. Tissue is routinely stored for at least 10 years by the Trust's histopathology department. Eventually all tissue is disposed of by incineration.

Should you specifically consent, samples will be frozen and stored for future research.

Is the information about me and my tissue treated confidentially?

Unless you have specifically given your consent for researchers or relevant regulatory authorities to access your medical records, complete confidentiality is guaranteed.

Data will be stored in a 'linked-anonymised' format, allowing only the Research Student and Principal investigator, using secure encrypted code, to identify you from your samples.

Where is tissue stored?

Tissue can be used for research as soon as it is taken from your body but it can also be stored for many years before it is used. Tissue collected for teaching and research is placed in a 'BioBank'. The Trust's Research BioBank is licensed by the Human Tissue Authority with Licence Number 11016 (http://www.hta.gov.uk/) and as such is strictly regulated.

Will I always know what research is being done on my tissue?

Unfortunately, it is not possible to tell you about all the specific research experiments that may use your tissue.

Could genetic research be performed on my tissue?

Yes. We will only be looking at genes known to be involved in Crohn's disease. We will not be able to identify genes involved in other diseases that could affect you or members of your family.

Could my tissue be used by any other organisations?

No.

What will happen if I do not want to carry on with the study?

If you no longer want your tissue to be used for research, you can withdraw your consent by contacting the research team. Any remaining tissue not already used in experiments will then be disposed of in an appropriate manner.

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

What happens to the results of the research?

We plan to publish the results in peer reviewed publications and we will inform subjects of this and forward copies if requested.

What if there is a problem or something goes wrong?

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated by members of staff you may have experienced due to your participation in the research, National Health Service or UCL complaints mechanisms are available to you. Please ask your research doctor if you would like more information on this.
In the unlikely event that you are harmed by taking part in this study, compensation may be available.
If you suspect that the harm is the result of the Sponsor's (University College London) or the hospital's negligence then you may be able to claim compensation. After discussing with your research doctor, please make the claim in writing to Professor Massimo Pinzani who is the Chief Investigator for the research and is based at UCL Institute for Liver and Digestive Health, University College London, Royal Free Hospital Campus U3, Rowland Hill Street, London NW3 2PF. The Chief Investigator will then pass the claim to the Sponsor's Insurers, via the Sponsor's office. You may have to bear the costs of the legal action initially, and you should consult a lawyer about this.

FUNDING

This research is being funded from the charitable Inflammatory Bowel Disease Research Fund:

Inflammatory Bowel Disease Research Fund
The Royal Free Charity
The Royal Free London NHS Foundation Trust
Pond Street
London NW3 2QG

Email: rfh.fundraising@nhs.net

MEMBERS OF THE RESEARCH TEAM

PhD Student:

Dr Thomas C Shepherd MBBS MRCP
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: tom.c.shepherd@me.com
Tel: 07739 136 838

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

Chief Investigator:

Professor Massimo Pinzani MD PhD FRCP
Sheila Sherlock Chair of Hepatology Director
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: m.pinzani@ucl.ac.uk

Investigators:

Dr Charles D Murray PhD FRCP
Consultant Gastroenterologist
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: charlesmurray1@nhs.net

Dr Krista Rombouts PhD
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: k.rombouts@ucl.ac.uk

Dr Pinelopi Manousou PhD
Sheila Sherlock Fellow
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: p.manousou@ucl.ac.uk

Appendix 5: Participant information sheet for patients who do not have Crohn's disease

1

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

Participant Information Sheet for Patients who do not have Crohn's Disease

---

Mechanisms of Intestinal Fibrosis in Stenotic Phenotypes of Crohn's Disease (Student Study)

INTRODUCTION

Royal Free London NHS Foundation Trust ('the Trust') is a leading teaching hospital and bio-medical centre, where many tissue and cell samples are taken from patients each day for laboratory tests. Examples of these specimens include blood samples, tissues removed during operations, and urine.

The Trust is a major centre for research into the causes of diseases and their treatment. For detailed information about our research, please visit our website: www.royalfree.nhs.uk

Invitation

You have been invited to take part in this study of Crohn's disease by donating gut tissue specimens and peripheral blood.

You have been identified as a potential healthy control (you do not have Crohn's disease, but you are having an endoscopy investigation of your gut).

Introduction to this research project

Crohn's disease (CD), a type of inflammatory bowel disease, causes inflammation along the entire digestive tract leading to disabling symptoms such as abdominal pain and bloody diarrhoea. The exact cause is unknown, but there is an abnormal response by the body's defences – immune system – and it attacks itself. Worldwide it affects anywhere between 5 to 10 people per 100,000 per year.

While there is no cure, modern medications that suppress the immune system have greatly improved patients' symptoms and reduced the need for surgical removal of diseased bowel by reducing inflammation. However, a proportion of patients will develop areas of narrowing in their bowel – strictures – that cause blockages. Modern medications do not treat strictures and patients often require multiple operations to remove them. Little is known about strictures in CD and why they develop.

Our research will focus on strictures to better understand how and why they develop, with the ultimate goal of proposing different and new treatments. Specifically, we will use strictured areas of bowel that have had to be surgically removed from patients with CD in addition to samples of their blood to investigate at a microscopic level the mechanisms involved in stricture development.

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

WHATS INVOLVED?

What is the purpose of this specific study?

Using samples of gut and blood we aim to research causes of gut narrowing (fibrosis) in inflammatory bowel disease, specifically Crohn's Disease, at a microscopic level.

Why have I been chosen to donate tissue and blood?

We are inviting to take part as you do NOT have Crohn's disease:

Patients who do not have Crohn's disease, but require endoscopic (colonoscopy or flexible sigmoidoscopy) investigation are being invited to donate biopsied tissue and a blood sample. Tissue and blood not affected by Crohn's disease will be compared to diseased tissue as a control.

What would taking part involve?

You have been approached as endoscopic investigation has been decided to be the most appropriate investigation as part of normal care.

As the study is laboratory based your direct involvement is limited, and as such, additional time, outside that required for normal standard of care, is small.

Pre-procedure consultation will allow you to ask any questions prior to informed consent being obtained.

Peripheral blood and tissue samples will be obtained at the time of your endoscopy for the purpose of our research study. A maximum of 10 millilitres of blood and 8 biopsies (tissue samples) will be taken in addition to what would be required as part of standard care.

Your endoscopy may appear normal to the naked eye, and sometimes biopsies would not routinely be taken. However, by consenting to take part in this research we will take a maximum of 8 biopsies that may then be used in our experiments. This number of biopsies will add approximately an extra 4 minutes on to the procedure time.

While your colon may have appeared normal to the naked eye, your biopsies could show an abnormality when analysed. If this is the case, we will not able to use them for the purpose of a normal control sample in our study, and they will be appropriately disposed of. This decision will be made by the Principal Investigator and PhD Research Student. You will be informed of any abnormality discovered as part of your standard of care medical follow up.

Subsequently, the investigator will not require direct patient contact.

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

Possible benefits

The nature of this research is such that immediate benefit to a specific participant is not anticipated. However, in time we hope results will contribute to benefit novel management in inflammatory bowel disease patients as a whole community.

We hope to further knowledge in Crohn's Disease that we would actively disseminate in an understandable format so patients, and the lay public at large, better understand the process of scarring with stricture formation and rationale for current treatment and the possibility of developing new therapies.

Possible disadvantages and risks

Additional burdens for research participants will be limited to time. You are required to spend non-pressurised time with this information leaflet, which will precede a consultation to answer potential questions and obtain informed consent. We will aim to limit additional visits to the hospital by incorporating this in to routine required hospital visits, although additional consultation time (above that of a normal outpatient visit) will be required.

Otherwise, adverse events will be associated with the standard of care in patients having endoscopic assessment of their bowel complaint, and these will not be enhanced by study participation.

The doctor performing your endoscopy will discus risks involved with your procedure. A flexible sigmoidoscopy with biopsies has a 1:15,000 chance of perforation (making a tear in the gut), while a colonoscopy with biopsies has a 1:1000 chance of perforation.

Consent

Consent will be obtained only by medically qualified members of the research team.

Patients identified as suitable candidates for donation, use and storage of human tissue and peripheral blood will be appropriately consented.

The independent consent form and discussion will be located in the patients source document (paper patient notes are in use at The Royal Free Hospital).

For non-English speaking participants the Royal Free Hospital has a telephone translation service in place and written information will be translated.

Every effort will be made to accommodate individual patients needs.

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

SUPPORTING INFORMATION

What will happen to the samples I give?

Donated tissue and blood samples will initially be frozen and stored. Subsequently they will be used in experiments for this research study. The experiments will take place in the medical laboratories located at The Royal Free Hospital.

What happens to your tissue after it has been used?

Samples of blood are only kept for a few weeks and are then disposed of by incineration. Tissue is routinely stored for at least 10 years by the Trust's histopathology department. Eventually all tissue is disposed of by incineration.

Should you specifically consent, samples will be frozen and stored for future research.

Is the information about me and my tissue treated confidentially?

Unless you have specifically given your consent for researchers or relevant regulatory authorities to access your medical records, complete confidentiality is guaranteed.

Data will be stored in a 'linked-anonymised' format, allowing only the Research Student and Principal investigator, using secure encrypted code, to identify you from your samples.

Where is tissue stored?

Tissue can be used for research as soon as it is taken from your body but it can also be stored for many years before it is used. Tissue collected for teaching and research is placed in a 'BioBank'. The Trust's Research BioBank is licensed by the Human Tissue Authority with Licence Number 11016 (http://www.hta.gov.uk/) and as such is strictly regulated.

Will I always know what research is being done on my tissue?

Unfortunately, it is not possible to tell you about all the specific research experiments that may use your tissue.

Could genetic research be performed on my tissue?

Yes. We will only be looking at genes known to be involved in Crohn's disease. We will not be able to identify genes involved in other diseases that could affect you or members of your family.

Could my tissue be used by any other organisations?

No.

What will happen if I do not want to carry on with the study?

If you no longer want your tissue to be used for research, you can withdraw your consent by contacting the research team. Any remaining tissue not already used in experiments will then be disposed of in an appropriate manner.

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

What happens to the results of the research?

We plan to publish the results in peer reviewed publications and we will inform subjects of this and forward copies if requested.

What if there is a problem or something goes wrong?

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated by members of staff you may have experienced due to your participation in the research, National Health Service or UCL complaints mechanisms are available to you. Please ask your research doctor if you would like more information on this.
In the unlikely event that you are harmed by taking part in this study, compensation may be available.
If you suspect that the harm is the result of the Sponsor's (University College London) or the hospital's negligence then you may be able to claim compensation. After discussing with your research doctor, please make the claim in writing to Professor Massimo Pinzani who is the Chief Investigator for the research and is based at UCL Institute for Liver and Digestive Health, University College London, Royal Free Hospital Campus U3, Rowland Hill Street, London NW3 2PF. The Chief Investigator will then pass the claim to the Sponsor's Insurers, via the Sponsor's office. You may have to bear the costs of the legal action initially, and you should consult a lawyer about this.

FUNDING

This research is being funded from the charitable Inflammatory Bowel Disease Research Fund:

Inflammatory Bowel Disease Research Fund
The Royal Free Charity
The Royal Free London NHS Foundation Trust
Pond Street
London NW3 2QG

Email: rfh.fundraising@nhs.net

MEMBERS OF THE RESEARCH TEAM

PhD Student: **Dr Thomas C Shepherd MBBS MRCP**
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: tom.c.shepherd@me.com
Tel: 07739 136 838

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

Chief Investigator:

Professor Massimo Pinzani MD PhD FRCP
Sheila Sherlock Chair of Hepatology Director
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: m.pinzani@ucl.ac.uk

Investigators:

Dr Charles D Murray PhD FRCP
Consultant Gastroenterologist
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: charlesmurray1@nhs.net

Dr Krista Rombouts PhD
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: k.rombouts@ucl.ac.uk

Dr Pinelopi Manousou PhD
Sheila Sherlock Fellow
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: p.manousou@ucl.ac.uk

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

INSURANCE AND INDEMNITY

University College London holds insurance against claims from participants for harm caused by their participation in this clinical study. Participants may be able to claim compensation if they can prove that UCL has been negligent. However, if this clinical study is being carried out in a hospital, the hospital continues to have a duty of care to the participant of the clinical study. University College London does not accept liability for any breach in the hospital's duty of care, or any negligence on the part of hospital employees. This applies whether the hospital is an NHS Trust or otherwise.

ETHICS

This study has been reviewed by the Riverside NRES Committee, who have granted a favourable opinion for this study to commence.

Appendix 6: Consent form for patients with Crohn's disease

1

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

Centre Number:

Study Number: 9005

Patient Identification Number for this study:

CONSENT FORM for patient's with Crohn's disease

Mechanisms of Intestinal Fibrosis in Stenotic Phenotypes of Crohn's Disease

(Student Study)

Name of Chief Investigator: **Professor Massimo Pinzani**

Please Initial All Boxes

1. I confirm that I have read and understand the **Participant Information Sheet dated 30th October 2015 (version 1.4)** 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, without my medical care or legal rights being affected; and that my tissue and blood samples will be appropriately disposed of. ☐
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 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 consent to donating tissue and blood samples for the above named project. ☐
5. I consent to the use of my samples for genetic analysis. ☐
6. I consent to my tissue and blood being stored securely by the Royal Free Hospital BioBank, and subsequently being used for future REC approved Inflammatory Bowel Disease research studies. ☐
7. I understand and agree to information and data obtained from work carried out on my tissue and blood may be used for reputable publication and presentation. ☐
8. I understand and agree to data relating to my donated samples being stored electronically. ☐

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

9. I understand that on no occasion will my identity be revealed without further explicit written consent. ☐

10. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

11. I agree to take part in the above study. ☐

Name of participant	Date	Signature

Name of person taking consent	Date	Signature

CONTACT:

PhD Student: **Dr Thomas C Shepherd MBBS MRCP**
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: tom.c.shepherd@me.com
Tel: 07739 136 838

Appendix 7: Consent form for patients who do not have Crohn's disease

1

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

Centre Number:

Study Number: 9005

Patient Identification Number for this study:

CONSENT FORM for patient's who do not have Crohn's disease

Mechanisms of Intestinal Fibrosis in Stenotic Phenotypes of Crohn's Disease

(Student Study)

Name of Chief Investigator: **Professor Massimo Pinzani**

Please Initial All Boxes

1. I confirm that I have read and understand the **Participant Information Sheet dated 30th October 2015 (version 1.3)** 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, without my medical care or legal rights being affected; and that my tissue and blood samples will be appropriately disposed of. ☐
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 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 consent to donating tissue and blood samples for the above named project. ☐
5. I consent to the use of my samples for genetic analysis. ☐
6. I consent to my tissue and blood being stored securely by the Royal Free Hospital BioBank, and subsequently being used for future REC approved Inflammatory Bowel Disease research studies. ☐
7. I understand and agree to information and data obtained from work carried out on my tissue and blood may be used for reputable publication and presentation. ☐
8. I understand and agree to data relating to my donated samples being stored electronically. ☐

Royal Free London NHS
NHS Foundation Trust

Royal Free Hospital
Pond Street
London
NW3 2QG

Tel: 020 3758 2000

9. I understand that on no occasion will my identity be revealed without further explicit written consent. ☐

10. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

11. I agree to take part in the above study. ☐

Name of participant	Date	Signature

Name of person taking consent	Date	Signature

CONTACT:

PhD Student: **Dr Thomas C Shepherd MBBS MRCP**
UCL Institute for Liver and Digestive Health
University College London
Royal Free Hospital Campus U3
Rowland Hill Street
London NW3 2PF
United Kingdom
Email: tom.c.shepherd@me.com
Tel: 07739 136 838

REFERENCES

1. Crohn BB, Ginzburg L, Oppenheimer GD. REGIONAL ILEITIS: A PATHOLOGIC AND CLINICAL ENTITY. *J Am Med Assoc*. 1932;99(16):1323-1329. doi:10.1001/JAMA.1932.02740680019005
2. Crohn BB. Granulomatous Diseases of the Small and Large Bowel: A historical survey. *Gastroenterology*. 1967;52(5):767-772. doi:10.1016/S0016-5085(67)80063-5
3. Luzoro A, Sabat P, Guzmán L, Frias F. Extraintestinal Manifestations of Inflammatory Bowel Disease. *Gastroenterol Hepatol (N Y)*. 2011;7(4):235. doi:10.1016/j.rmclc.2019.06.001
4. Baumgart DC, Sandborn WJ. Crohn's disease. *Lancet*. 2012;380(9853):1590-1605. doi:10.1016/S0140-6736(12)60026-9
5. Pasvol TJ, Horsfall L, Bloom S, et al. Original research: Incidence and prevalence of inflammatory bowel disease in UK primary care: a population-based cohort study. *BMJ Open*. 2020;10(7):36584. doi:10.1136/BMJOPEN-2019-036584
6. Burisch J, Jess T, Martinato M, Lakatos PL. The burden of inflammatory bowel disease in Europe. *J Crohns Colitis*. 2013;7(4):322-337. doi:10.1016/J.CROHNS.2013.01.010
7. Luces C, Bodger K. Economic burden of inflammatory bowel disease: a UK perspective. *Expert Rev Pharmacoecon Outcomes Res*. 2006;6(4):471-482. doi:10.1586/14737167.6.4.471
8. Gunesh S, Thomas GAO, Williams GT, Roberts A, Hawthorne AB. The incidence of Crohn's disease in Cardiff over the last 75 years: An update for 1996-2005. *Aliment Pharmacol Ther*. 2008;27(3):211-219. doi:10.1111/j.1365-2036.2007.03576.x
9. Logan RFA. Inflammatory bowel disease incidence: Up, down or unchanged? *Gut*. 1998;42(3):309-311. doi:10.1136/gut.42.3.309
10. M'Koma AE. Inflammatory Bowel Disease: An Expanding Global Health Problem. *Clin Med Insights Gastroenterol*. 2013;6(6):33. doi:10.4137/CGAST.S12731
11. King D, Reulen RC, Thomas T, et al. Changing patterns in the epidemiology and outcomes of inflammatory bowel disease in the United

Kingdom: 2000-2018. *Aliment Pharmacol Ther*. 2020;51(10):922-934. doi:10.1111/APT.15701
12. Hamilton B, Green H, Heerasing N, et al. Original research: Incidence and prevalence of inflammatory bowel disease in Devon, UK. *Frontline Gastroenterol*. 2021;12(6):461. doi:10.1136/FLGASTRO-2019-101369
13. Lakatos PL. Recent trends in the epidemiology of inflammatory bowel diseases: Up or down? *World Journal of Gastroenterology : WJG*. 2006;12(38):6102. doi:10.3748/WJG.V12.I38.6102
14. Silverberg MS, Satsangi J, Ahmad T, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol*. 2005;19 Suppl A. doi:10.1155/2005/269076
15. Hendrickson BA, Gokhale R, Cho JH. Clinical Aspects and Pathophysiology of Inflammatory Bowel Disease. *Clin Microbiol Rev*. 2002;15(1):79. doi:10.1128/CMR.15.1.79-94.2002
16. Hedrick T, Friel C. Colonic Crohn Disease. *Clin Colon Rectal Surg*. 2013;26(2):84. doi:10.1055/S-0033-1348046
17. Louis E, Michel V, Hugot JP, et al. Early development of stricturing or penetrating pattern in Crohn's disease is influenced by disease location, number of flares, and smoking but not by NOD2/CARD15 genotype. *Gut*. 2003;52(4):552. doi:10.1136/GUT.52.4.552
18. Lin X, Wang Y, Liu Z, et al. Intestinal strictures in Crohn's disease: a 2021 update. *Therap Adv Gastroenterol*. 2022;15. doi:10.1177/17562848221104951
19. Chang CW, Wong JM, Tung CC, Shih IL, Wang HY, Wei SC. Intestinal Stricture in Crohn's Disease. *Intest Res*. 2015;13(1):19. doi:10.5217/IR.2015.13.1.19
20. Louis E, Collard A, Oger AF, Degroote E, Aboul Nasr El Yafi F, Belaiche J. Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. *Gut*. 2001;49(6):777-782. doi:10.1136/GUT.49.6.777
21. Gupta N, Bostrom AG, Kirschner BS, et al. Incidence of Stricturing and Penetrating Complications of Crohn's Disease Diagnosed in Pediatric Patients. *Inflamm Bowel Dis*. 2010;16(4):638. doi:10.1002/IBD.21099

22. Cosnes J, Cattan S, Blain A, et al. Long-term evolution of disease behavior of Crohn's disease. *Inflamm Bowel Dis*. 2002;8(4):244-250. doi:10.1097/00054725-200207000-00002
23. Thia KT, Sandborn WJ, Harmsen WS, Zinsmeister AR, Loftus E V. Risk Factors Associated With Progression to Intestinal Complications of Crohn's Disease in a Population-Based Cohort. *Gastroenterology*. 2010;139(4):1147. doi:10.1053/J.GASTRO.2010.06.070
24. Hanauer SB, Feagan BG, Lichtenstein GR, et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet*. 2002;359(9317):1541-1549. doi:10.1016/S0140-6736(02)08512-4
25. Lichtenstein GR. The TREAT Registry: Evolution of Knowledge From 1999 to 2017: Lessons Learned. *Clin Gastroenterol Hepatol*. 2017;15(9):1319-1321. doi:10.1016/J.CGH.2017.05.035
26. Rieder F, Brenmoehl J, Leeb S, Schölmerich J, Rogler G. Wound healing and fibrosis in intestinal disease. *Gut*. 2007;56(1):130. doi:10.1136/GUT.2006.090456
27. Alourfi M, Mosli M, Saadah OI. The Efficacy of Anti-Tumor Necrosis Factor Alpha for Symptomatic Stricturing Small Bowel Crohn's Disease. *Cureus*. 2020;12(9). doi:10.7759/CUREUS.10315
28. Vuyyuru SK, Kante B, Kumar P, et al. Real world analysis on the efficacy and safety of anti-tumor necrosis factor therapy in patients with stricturing Crohn's disease. *Sci Rep*. 2021;11(1):11704. doi:10.1038/S41598-021-90660-2
29. O'Sullivan S, Gilmer JF, Medina C. Matrix Metalloproteinases in Inflammatory Bowel Disease: An Update. *Mediators Inflamm*. 2015;2015. doi:10.1155/2015/964131
30. Mäkitalo L, Sipponen T, Kärkkäinen P, Kolho KL, Saarialho-Kere U. Changes in matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinases (TIMP) expression profile in Crohn's disease after immunosuppressive treatment correlate with histological score and calprotectin values. *Int J Colorectal Dis*. 2009;24(10):1157-1167. doi:10.1007/S00384-009-0756-5
31. Yoo JH, Holubar S, Rieder F. Fibrostenotic strictures in Crohn's disease. *Intest Res*. 2020;18(4):379. doi:10.5217/IR.2019.09148

32. Schmiedlin-Ren P, Reingold LJ, Broxson CS, et al. Anti-TNFα alters the natural history of experimental Crohn's disease in rats when begun early, but not late, in disease. *Am J Physiol Gastrointest Liver Physiol*. 2016;311(4):G688. doi:10.1152/AJPGI.00216.2015
33. Billmeier U, Dieterich W, Neurath MF, Atreya R. Molecular mechanism of action of anti-tumor necrosis factor antibodies in inflammatory bowel diseases. *World J Gastroenterol*. 2016;22(42):9300. doi:10.3748/WJG.V22.I42.9300
34. Ismail MS, Charabaty A. Management of Crohn's stricture: medical, endoscopic and surgical therapies. *Frontline Gastroenterol*. 2022;13(6):524-530. doi:10.1136/FLGASTRO-2021-101827
35. Büning C, Lochs H. Conventional therapy for Crohn's disease. *World Journal of Gastroenterology : WJG*. 2006;12(30):4794. doi:10.3748/WJG.V12.I30.4794
36. Caio G, Lungaro L, Caputo F, et al. Nutritional Treatment in Crohn's Disease. *Nutrients*. 2021;13(5). doi:10.3390/NU13051628
37. Carter MJ, Lobo AJ, Travis SPL. Guidelines for the management of inflammatory bowel disease in adults. *Gut*. 2004;53(suppl 5):v1-v16. doi:10.1136/GUT.2004.043372
38. Sulz MC, Burri E, Michetti P, Rogler G, Peyrin-Biroulet L, Seibold F. Treatment Algorithms for Crohn's Disease. *Digestion*. 2020;101(Suppl. 1):43-57. doi:10.1159/000506364
39. Hanauer SB, Strömberg U. Oral pentasa in the treatment of active Crohn's disease: A meta-analysis of double-blind, placebo-controlled trials. *Clinical Gastroenterology and Hepatology*. 2004;2(5):379-388. doi:10.1016/S1542-3565(04)00122-3
40. Cushing K, Higgins PDR. Management of Crohn Disease: A Review. *JAMA*. 2021;325(1):69. doi:10.1001/JAMA.2020.18936
41. Chande N, Townsend CM, Parker CE, Macdonald JK. Azathioprine or 6-mercaptopurine for induction of remission in Crohn's disease. *Cochrane Database Syst Rev*. 2016;2016(10). doi:10.1002/14651858.CD000545.PUB5
42. Nielsen OH, Steenholdt C, Juhl CB, Rogler G. Efficacy and safety of methotrexate in the management of inflammatory bowel disease: A

systematic review and meta-analysis of randomized, controlled trials. *EClinicalMedicine*. 2020;20. doi:10.1016/J.ECLINM.2020.100271
43. Adegbola SO, Sahnan K, Warusavitarne J, Hart A, Tozer P. Anti-TNF Therapy in Crohn's Disease. *Int J Mol Sci*. 2018;19(8). doi:10.3390/IJMS19082244
44. Lam MCW, Bressler B. Vedolizumab for ulcerative colitis and Crohn's disease: results and implications of GEMINI studies. *Immunotherapy*. 2014;6(9):963-971. doi:10.2217/IMT.14.66
45. Zingone F, Barberio B, Compostella F, et al. Good efficacy and safety of vedolizumab in Crohn's disease and ulcerative colitis in a real-world scenario. *Therap Adv Gastroenterol*. 2020;13. doi:10.1177/1756284820936536
46. Harris RJ, McDonnell M, Young D, et al. Early real-world effectiveness of ustekinumab for Crohn's disease. *Frontline Gastroenterol*. 2020;11(2):111-116. doi:10.1136/FLGASTRO-2019-101237
47. Ferrante M, Panaccione R, Baert F, et al. Risankizumab as maintenance therapy for moderately to severely active Crohn's disease: results from the multicentre, randomised, double-blind, placebo-controlled, withdrawal phase 3 FORTIFY maintenance trial. *Lancet*. 2022;399(10340):2031-2046. doi:10.1016/S0140-6736(22)00466-4
48. Loftus E V, Panés J, Lacerda AP, et al. Upadacitinib Induction and Maintenance Therapy for Crohn's Disease. *N Engl J Med*. 2023;388(21):1966-1980. doi:10.1056/NEJMOA2212728/SUPPL_FILE/NEJMOA2212728_DATA-SHARING.PDF
49. Kumar A, Cole A, Segal J, Smith P, Limdi JK. A review of the therapeutic management of Crohn's disease. *Therap Adv Gastroenterol*. 2022;15. doi:10.1177/17562848221078456
50. Albader F, Golovics PA, Gonczi L, Bessissow T, Afif W, Lakatos PL. Therapeutic drug monitoring in inflammatory bowel disease: The dawn of reactive monitoring. *World J Gastroenterol*. 2021;27(37):6231. doi:10.3748/WJG.V27.I37.6231
51. Papamichael K, Vande Casteele N, Jeyarajah J, Jairath V, Osterman MT, Cheifetz AS. Higher Postinduction Infliximab Concentrations Are Associated With Improved Clinical Outcomes in Fistulizing Crohn's

Disease: An ACCENT-II Post Hoc Analysis. *Am J Gastroenterol*. 2021;116(5):1007-1014. doi:10.14309/AJG.0000000000001111

52. Vizuete JA, Randall CW, Taboada CM, et al. From historical perspectives to modern therapy: a review of current and future biological treatments for Crohn's disease. *Therap Adv Gastroenterol*. 2015;8(3):143. doi:10.1177/1756283X15576462
53. Lu C, Baraty B, Lee Robertson H, et al. Systematic Review: Medical Therapy for Fibrostenosing Crohn's Disease. *Aliment Pharmacol Ther*. 2020;51(12):1233. doi:10.1111/APT.15750
54. Sleiman J, El Ouali S, Qazi T, et al. Prevention and treatment of stricturing Crohn's disease – perspectives and challenges. *Expert Rev Gastroenterol Hepatol*. 2021;15(4):401. doi:10.1080/17474124.2021.1854732
55. Bernell O, Lapidus A, Hellers G. Risk Factors for Surgery and Postoperative Recurrence in Crohn's Disease. *Ann Surg*. 2000;231(1):38. doi:10.1097/00000658-200001000-00006
56. Peyrin-Biroulet L, Harmsen WS, Tremaine WJ, Zinsmeister AR, Sandborn WJ, Loftus E V. SURGERY IN A POPULATION-BASED COHORT OF CROHN'S DISEASE FROM OLMSTED COUNTY, MINNESOTA (1970–2004). *Am J Gastroenterol*. 2012;107(11):1693. doi:10.1038/AJG.2012.298
57. Gklavas A, Dellaportas D, Papaconstantinou I. Risk factors for postoperative recurrence of Crohn's disease with emphasis on surgical predictors. *Ann Gastroenterol*. 2017;30(6):598. doi:10.20524/AOG.2017.0195
58. Latella G, Rogler G, Bamias G, et al. Results of the 4th scientific workshop of the ECCO (I): pathophysiology of intestinal fibrosis in IBD. *J Crohns Colitis*. 2014;8(10):1147-1165. doi:10.1016/J.CROHNS.2014.03.008
59. Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut*. 2006;55(6):749. doi:10.1136/GUT.2005.082909
60. Rieder F, Zimmermann EM, Remzi FH, Sandborn WJ. Crohn's disease complicated by strictures: a systematic review. *Gut*. 2013;62(7):1072. doi:10.1136/GUTJNL-2012-304353
61. Pak W, Chan W, Mourad F, et al. Crohn's disease associated strictures. *J Gastroenterol Hepatol*. 2018;33(5):998-1008. doi:10.1111/JGH.14119

62. Fukumoto A, Tanaka S, Yamamoto H, et al. Diagnosis and treatment of small-bowel stricture by double balloon endoscopy. *Gastrointest Endosc*. 2007;66(3 Suppl). doi:10.1016/J.GIE.2007.02.027
63. Gasche C, Scholmerich J, Brynskov J, et al. A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. *Inflamm Bowel Dis*. 2000;6(1):8-15. doi:10.1097/00054725-200002000-00002
64. Nikolaus S, Schreiber S. Diagnostics of inflammatory bowel disease. *Gastroenterology*. 2007;133(5):1670-1689. doi:10.1053/J.GASTRO.2007.09.001
65. Freeman HJ. Natural history and clinical behavior of Crohn's disease extending beyond two decades. *J Clin Gastroenterol*. 2003;37(3):216-219. doi:10.1097/00004836-200309000-00005
66. El Ouali S, Click B, Holubar SD, Rieder F. Natural history, diagnosis and treatment approach to fibrostenosing Crohn's disease. *United European Gastroenterol J*. 2020;8(3):263. doi:10.1177/2050640620901960
67. Rieder F, Bettenworth D, Ma C, et al. An expert consensus to standardize definitions, diagnosis and treatment targets for anti-fibrotic stricture therapies in Crohn's disease. *Aliment Pharmacol Ther*. 2018;48(3):347. doi:10.1111/APT.14853
68. Villanacci V, Reggiani-Bonetti L, Salviato T, et al. Histopathology of IBD Colitis. A practical approach from the pathologists of the Italian Group for the study of the gastrointestinal tract (GIPAD). *Pathologica*. 2021;113(1):39. doi:10.32074/1591-951X-235
69. Fumery M, Pineton de Chambrun G, Stefanescu C, et al. Detection of Dysplasia or Cancer in 3.5% of Patients With Inflammatory Bowel Disease and Colonic Strictures. *Clin Gastroenterol Hepatol*. 2015;13(10):1770-1775. doi:10.1016/J.CGH.2015.04.185
70. Gordon IO, Bettenworth D, Bokemeyer A, et al. Histopathology Scoring Systems of Stenosis Associated With Small Bowel Crohn's Disease: A Systematic Review. *Gastroenterology*. 2020;158(1):137. doi:10.1053/J.GASTRO.2019.08.033
71. Rao N, Kumar S, Taylor S, Plumb A. Diagnostic pathways in Crohn's disease. *Clin Radiol*. 2019;74(8):578-591. doi:10.1016/j.crad.2019.03.013

72. A global consensus on the definitions, diagnosis and management of fibrostenosing small bowel Crohn's disease in clinical practice | Enhanced Reader.
73. Santacroce G, Lenti MV, Di Sabatino A. Therapeutic Targeting of Intestinal Fibrosis in Crohn's Disease. *Cells*. 2022;11(3). doi:10.3390/CELLS11030429
74. Hirai F. Current status of endoscopic balloon dilation for Crohn's disease. *Intest Res*. 2017;15(2):166. doi:10.5217/IR.2017.15.2.166
75. Morar PS, Faiz O, Warusavitarne J, et al. Systematic review with meta-analysis: endoscopic balloon dilatation for Crohn's disease strictures. *Aliment Pharmacol Ther*. 2015;42(10):1137-1148. doi:10.1111/APT.13388
76. Chandrasinghe P. Surgical Management of Small Bowel Crohn's Disease. *Front Surg*. 2022;9:759668. doi:10.3389/FSURG.2022.759668/BIBTEX
77. Ambe R, Campbell L, Cagir B. A comprehensive review of strictureplasty techniques in Crohn's disease: types, indications, comparisons, and safety. *J Gastrointest Surg*. 2012;16(1):209-217. doi:10.1007/S11605-011-1651-2
78. Butt WT, Ryan ÉJ, Boland MR, et al. Strictureplasty versus bowel resection for the surgical management of fibrostenotic Crohn's disease: a systematic review and meta-analysis. *Int J Colorectal Dis*. 2020;35(4):705-717. doi:10.1007/S00384-020-03507-Z
79. Nos P, Domènech E. Postoperative Crohn's disease recurrence: A practical approach. *World Journal of Gastroenterology : WJG*. 2008;14(36):5540. doi:10.3748/WJG.14.5540
80. Rutgeerts P, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology*. 1990;99(4):956-963. doi:10.1016/0016-5085(90)90613-6
81. Li C, Kuemmerle JF. Mechanisms that mediate the development of fibrosis in patients with Crohn's disease. *Inflamm Bowel Dis*. 2014;20(7):1250-1258. doi:10.1097/MIB.0000000000000043
82. Bettenworth D, Rieder F. Pathogenesis of Intestinal Fibrosis in Inflammatory Bowel Disease and Perspectives for Therapeutic Implication. *Dig Dis*. 2017;35(1-2):25-31. doi:10.1159/000449079

83. Rieder F, Fiocchi C, Rogler G. Mechanisms, Management, and Treatment of Fibrosis in Patients With Inflammatory Bowel Diseases. *Gastroenterology*. 2017;152(2):340-350.e6. doi:10.1053/J.GASTRO.2016.09.047
84. Rieder F. Managing Intestinal Fibrosis in Patients With Inflammatory Bowel Disease. *Gastroenterol Hepatol (N Y)*. 2018;14(2):120. Accessed July 16, 2023. /pmc/articles/PMC5866304/
85. Latella G, Di Gregorio J, Flati V, Rieder F, Lawrance IC. Mechanisms of initiation and progression of intestinal fibrosis in IBD. *Scand J Gastroenterol*. 2015;50(1):53-65. doi:10.3109/00365521.2014.968863
86. Pucilowska JB, Williams KL, Lund PK. Fibrogenesis. IV. Fibrosis and inflammatory bowel disease: cellular mediators and animal models. *Am J Physiol Gastrointest Liver Physiol*. 2000;279(4). doi:10.1152/AJPGI.2000.279.4.G653
87. Burke JP, Mulsow JJ, O'Keane C, Docherty NG, Watson RWG, O'Connell PR. Fibrogenesis in Crohn's disease. *Am J Gastroenterol*. 2007;102(2):439-448. doi:10.1111/J.1572-0241.2006.01010.X
88. Rieder F, Fiocchi C. Intestinal fibrosis in IBD—a dynamic, multifactorial process. *Nature Reviews Gastroenterology & Hepatology 2009 6:4*. 2009;6(4):228-235. doi:10.1038/nrgastro.2009.31
89. Derkacz A, Olczyk P, Olczyk K, Komosinska-Vassev K. The Role of Extracellular Matrix Components in Inflammatory Bowel Diseases. *Journal of Clinical Medicine 2021, Vol 10, Page 1122*. 2021;10(5):1122. doi:10.3390/JCM10051122
90. Rieder F, Bettenworth D, Imai J, Inagaki Y. Intestinal Fibrosis and Liver Fibrosis: Consequences of Chronic Inflammation or Independent Pathophysiology? *Inflamm Intest Dis*. 2016;1(1):41. doi:10.1159/000445135
91. Trépo C, Chan HLY, Lok A. Hepatitis B virus infection. *Lancet*. 2014;384(9959):2053-2063. doi:10.1016/S0140-6736(14)60220-8
92. Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med*. 2001;345(1):41-52. doi:10.1056/NEJM200107053450107
93. Suda K. Pathogenesis and progression of human pancreatic fibrosis. *Med Electron Microsc*. 2000;33(4):200-206. doi:10.1007/S007950000020

94. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med*. 2012;18(7):1028-1040. doi:10.1038/NM.2807
95. Alfredsson J, Wick MJ. Mechanism of fibrosis and stricture formation in Crohn's disease. *Scand J Immunol*. 2020;92(6). doi:10.1111/SJI.12990
96. Rieder F, Karrasch T, Ben-Horin S, et al. Results of the 2nd scientific workshop of the ECCO (III): basic mechanisms of intestinal healing. *J Crohns Colitis*. 2012;6(3):373-385. doi:10.1016/J.CROHNS.2011.11.009
97. Fiocchi C, Kay Lund P. Themes in fibrosis and gastrointestinal inflammation. *Am J Physiol Gastrointest Liver Physiol*. 2011;300(5). doi:10.1152/AJPGI.00104.2011
98. Latella G, Sferra R, Speca S, Vetuschi A, Gaudio E, Latella G. Can we prevent, reduce or reverse intestinal fibrosis in IBD? *Eur Rev Med Pharmacol Sci*. 2013;17(10):1283-1304.
99. McKaig BC, McWilliams D, Watson SA, Mahida YR. Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. *Am J Pathol*. 2003;162(4):1355-1360. doi:10.1016/S0002-9440(10)63931-4
100. Murphy G, Nagase H. Progress in matrix metalloproteinase research. *Mol Aspects Med*. 2008;29(5):290-308. doi:10.1016/J.MAM.2008.05.002
101. Warnaar N, Hofker HS, Maathuis MHJ, et al. Matrix metalloproteinases as profibrotic factors in terminal ileum in Crohn's disease. *Inflamm Bowel Dis*. 2006;12(9):863-869. doi:10.1097/01.MIB.0000231568.43065.ED
102. Barron L, Wynn TA. Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages. *Am J Physiol Gastrointest Liver Physiol*. 2011;300(5):G723. doi:10.1152/AJPGI.00414.2010
103. Pinchuk I V., Mifflin RC, Saada JI, Powell DW. Intestinal mesenchymal cells. *Curr Gastroenterol Rep*. 2010;12(5):310-318. doi:10.1007/S11894-010-0135-Y
104. Leeb SN, Vogl D, Grossmann J, et al. Autocrine fibronectin-induced migration of human colonic fibroblasts. *Am J Gastroenterol*. 2004;99(2):335-340. doi:10.1111/J.1572-0241.2004.04044.X

105. Leeb SN, Vogl D, Gunckel M, et al. Reduced Migration of Fibroblasts in Inflammatory Bowel Disease: Role of Inflammatory Mediators and Focal Adhesion Kinase. *Gastroenterology*. 2003;125(5):1341-1354. doi:10.1016/j.gastro.2003.07.004
106. Drygiannakis I, Valatas V, Sfakianaki O, et al. Proinflammatory cytokines induce crosstalk between colonic epithelial cells and subepithelial myofibroblasts: implication in intestinal fibrosis. *J Crohns Colitis*. 2013;7(4):286-300. doi:10.1016/J.CROHNS.2012.04.008
107. Rieder F, Schirbel A, Ouyang Z, et al. 192 Pro-Fibrogenic Activity of Toll-Like Receptor (TLR) and NOD-Like Receptor (NLR) Ligands on Human Intestinal Myofibroblasts (HIF) – Linking Bacterial Innate Immunity to Intestinal Fibrosis. *Gastroenterology*. 2010;138(5):S-35. doi:10.1016/S0016-5085(10)60163-1
108. Wengrower D, Zanninelli G, Latella G, et al. Losartan reduces trinitrobenzene sulphonic acid-induced colorectal fibrosis in rats. *Can J Gastroenterol*. 2012;26(1):33-39. doi:10.1155/2012/628268
109. Wengrower D, Zannineli G, Pappo O, et al. Prevention of Fibrosis in Experimental Colitis by Captopril: the Role of tgf-β1. *Inflamm Bowel Dis*. 2004;10(5):536-545. doi:10.1097/00054725-200409000-00007
110. Graham MF, Diegelmann RF, Elson CO, et al. Collagen content and types in the intestinal strictures of Crohn's disease. *Gastroenterology*. 1988;94(2):257-265. doi:10.1016/0016-5085(88)90411-8
111. Flynn RS, Murthy KS, Grider JR, Kellum JM, Kuemmerle JF. Endogenous IGF-I and alphaVbeta3 integrin ligands regulate increased smooth muscle hyperplasia in stricturing Crohn's disease. *Gastroenterology*. 2010;138(1):285-293. doi:10.1053/J.GASTRO.2009.09.003
112. Burke JP, Cunningham MF, Sweeney C, Docherty NG, O'Connell PR. N-cadherin is overexpressed in Crohn's stricture fibroblasts and promotes intestinal fibroblast migration. *Inflamm Bowel Dis*. 2011;17(8):1665-1673. doi:10.1002/IBD.21543
113. Yan X, Liu Z, Chen Y. Regulation of TGF-beta signaling by Smad7. *Acta Biochim Biophys Sin (Shanghai)*. 2009;41(4):263-272. doi:10.1093/ABBS/GMP018
114. Mckaig BC, Hughes K, Tighe PJ, Mahida AYR. Differential expression of TGF-beta isoforms by normal and inflammatory bowel disease intestinal

myofibroblasts. *Am J Physiol Cell Physiol*. 2002;282(1). doi:10.1152/AJPCELL.00048.2001
115. Lakatos G, Hritz I, Varga MZ, et al. The impact of matrix metalloproteinases and their tissue inhibitors in inflammatory bowel diseases. *Dig Dis*. 2012;30(3):289-295. doi:10.1159/000336995
116. Cabral-Pacheco GA, Garza-Veloz I, Rosa CCD La, et al. The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases. *Int J Mol Sci*. 2020;21(24):1-53. doi:10.3390/IJMS21249739
117. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res*. 2006;69(3):562-573. doi:10.1016/J.CARDIORES.2005.12.002
118. Barrett JC, Hansoul S, Nicolae DL, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet*. 2008;40(8):955-962. doi:10.1038/NG.175
119. Brant SR. Promises, delivery, and challenges of inflammatory bowel disease risk gene discovery. *Clin Gastroenterol Hepatol*. 2013;11(1):22-26. doi:10.1016/J.CGH.2012.11.001
120. Cho JH, Brant SR. Recent insights into the genetics of inflammatory bowel disease. *Gastroenterology*. 2011;140(6):1704-1712.e2. doi:10.1053/J.GASTRO.2011.02.046
121. Franke A, McGovern DPB, Barrett JC, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet*. 2010;42(12):1118-1125. doi:10.1038/NG.717
122. Rieder F, Lawrance IC, Leite A, Sans M. Predictors of fibrostenotic Crohn's disease. *Inflamm Bowel Dis*. 2011;17(9):2000-2007. doi:10.1002/IBD.21627
123. Hume GE, Fowler E V., Lincoln D, et al. Angiotensinogen and transforming growth factor beta1: novel genes in the pathogenesis of Crohn's disease. *J Med Genet*. 2006;43(10). doi:10.1136/JMG.2005.040477
124. Meijer MJW, Mieremet-Ooms MAC, van Hogezand RA, Lamers CBHW, Hommes DW, Verspaget HW. Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-alpha single nucleotide gene polymorphisms in inflammatory bowel disease. *World J Gastroenterol*. 2007;13(21):2960-2966. doi:10.3748/WJG.V13.I21.2960

125. Lord JD. Promises and paradoxes of regulatory T cells in inflammatory bowel disease. *World Journal of Gastroenterology : WJG*. 2015;21(40):11236. doi:10.3748/WJG.V21.I40.11236
126. X Y, JJ L, RJ L, et al. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J*. 1999;18(5):1280-1291. doi:10.1093/EMBOJ/18.5.1280
127. Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y, Wang XF. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Mol Cell Biol*. 1999;19(4):2495-2504. doi:10.1128/MCB.19.4.2495
128. Latella G, Vetuschi A, Sferra R, et al. Smad3 loss confers resistance to the development of trinitrobenzene sulfonic acid-induced colorectal fibrosis. *Eur J Clin Invest*. 2009;39(2):145-156. doi:10.1111/J.1365-2362.2008.02076.X
129. Flynn RS, Mahavadi S, Murthy KS, et al. Endogenous IGFBP-3 regulates excess collagen expression in intestinal smooth muscle cells of Crohn's disease strictures. *Inflamm Bowel Dis*. 2011;17(1):193-201. doi:10.1002/IBD.21351
130. Katsumoto TR, Violette SM, Sheppard D. Blocking TGFβ via Inhibition of the αvβ6 Integrin: A Possible Therapy for Systemic Sclerosis Interstitial Lung Disease. *Int J Rheumatol*. 2011;2011. doi:10.1155/2011/208219
131. Klopcic B, Appelbee A, Raye W, et al. Indomethacin and retinoic acid modify mouse intestinal inflammation and fibrosis: a role for SPARC. *Dig Dis Sci*. 2008;53(6):1553-1563. doi:10.1007/S10620-007-0068-Y
132. Paul J, Singh AK, Kathania M, et al. IL-17-driven intestinal fibrosis is inhibited by Itch-mediated ubiquitination of HIC-5. *Mucosal Immunol*. 2018;11(2):427-436. doi:10.1038/MI.2017.53
133. Zhu Z, Li M, Shu X, et al. Thalidomide is a therapeutic agent that is effective in inducing and maintaining endoscopic remission in adult CD patients. *Clin Res Hepatol Gastroenterol*. 2017;41(2):210-216. doi:10.1016/J.CLINRE.2016.09.012
134. Chen H, Xu H, Luo L, et al. Thalidomide Prevented and Ameliorated Pathogenesis of Crohn's Disease in Mice via Regulation of Inflammatory Response and Fibrosis. *Front Pharmacol*. 2019;10. doi:10.3389/FPHAR.2019.01486

135. Zhao X, Chen J, Sun H, Zhang Y, Zou D. New insights into fibrosis from the ECM degradation perspective: the macrophage-MMP-ECM interaction. *Cell Biosci*. 2022;12(1). doi:10.1186/S13578-022-00856-W
136. Rieder F, Latella G, Magro F, et al. European Crohn's and Colitis Organisation Topical Review on Prediction, Diagnosis and Management of Fibrostenosing Crohn's Disease. *J Crohns Colitis*. 2016;10(8):873-885. doi:10.1093/ECCO-JCC/JJW055
137. Zittan E, Gralnek IM, Berns MS. The New Proactive Approach and Precision Medicine in Crohn's Disease. *Biomedicines*. 2020;8(7). doi:10.3390/BIOMEDICINES8070193
138. Mahida YR, Galvin AM, Gray T, et al. Migration of human intestinal lamina propria lymphocytes, macrophages and eosinophils following the loss of surface epithelial cells. *Clin Exp Immunol*. 1997;109(2):377-386. doi:10.1046/J.1365-2249.1997.4481346.X
139. Khalil H, Nie W, Edwards RA, Yoo J. Isolation of Primary Myofibroblasts from Mouse and Human Colon Tissue. *J Vis Exp*. 2013;80(80):50611. doi:10.3791/50611
140. Di Sabatino A, Pender SLF, Jackson CL, et al. Functional Modulation of Crohn's Disease Myofibroblasts by Anti-Tumor Necrosis Factor Antibodies. *Gastroenterology*. 2007;133(1):137-149. doi:10.1053/j.gastro.2007.04.069
141. Eyden B. The myofibroblast: phenotypic characterization as a prerequisite to understanding its functions in translational medicine. *J Cell Mol Med*. 2008;12(1):22. doi:10.1111/J.1582-4934.2007.00213.X
142. Li S, Lu R, Shu L, et al. An integrated map of fibroblastic populations in human colon mucosa and cancer tissues. *Commun Biol*. 2022;5(1):1326. doi:10.1038/S42003-022-04298-5
143. Cecchini MJ, Amiri M, Dick FA. Analysis of cell cycle position in mammalian cells. *J Vis Exp*. 2012;(59):1-7. doi:10.3791/3491
144. Nagar S, Hanley-Bowdoin L, Robertson D. Host DNA replication is induced by geminivirus infection of differentiated plant cells. *Plant Cell*. 2002;14(12):2995-3007. doi:10.1105/TPC.005777
145. Mahmood T, Yang PC. Western Blot: Technique, Theory, and Trouble Shooting. *N Am J Med Sci*. 2012;4(9):429. doi:10.4103/1947-2714.100998

146. Marrone G, De Chiara F, Böttcher K, et al. The adenosine monophosphate-activated protein kinase-vacuolar adenosine triphosphatase-pH axis: A key regulator of the profibrogenic phenotype of human hepatic stellate cells. *Hepatology*. 2018;68(3):1140-1153. doi:10.1002/HEP.30029
147. De Chiara F, Thomsen KL, Habtesion A, et al. Ammonia Scavenging Prevents Progression of Fibrosis in Experimental Nonalcoholic Fatty Liver Disease. *Hepatology*. 2020;71(3):874-892. doi:10.1002/HEP.30890
148. Bottcher K, Longato L, Marrone G, et al. AICAR and compound C negatively modulate HCC-induced primary human hepatic stellate cell activation in vitro. *Am J Physiol Gastrointest Liver Physiol*. 2021;320(4):G543-G556. doi:10.1152/AJPGI.00262.2020
149. Laskaratos FM, Levi A, Schwach G, et al. Transcriptomic Profiling of In Vitro Tumor-Stromal Cell Paracrine Crosstalk Identifies Involvement of the Integrin Signaling Pathway in the Pathogenesis of Mesenteric Fibrosis in Human Small Intestinal Neuroendocrine Neoplasms. *Front Oncol*. 2021;11. doi:10.3389/FONC.2021.629665
150. Dowling P, Clynes M. Conditioned media from cell lines: a complementary model to clinical specimens for the discovery of disease-specific biomarkers. *Proteomics*. 2011;11(4):794-804. doi:10.1002/PMIC.201000530
151. Shen C, Lie P, Miao T, et al. Conditioned medium from umbilical cord mesenchymal stem cells induces migration and angiogenesis. *Mol Med Rep*. 2015;12(1):20-30. doi:10.3892/MMR.2015.3409
152. Webber J, Jenkins RH, Meran S, Phillips A, Steadman R. Modulation of TGFβ1-Dependent Myofibroblast Differentiation by Hyaluronan. *Am J Pathol*. 2009;175(1):148. doi:10.2353/AJPATH.2009.080837
153. Midgley AC, Rogers M, Hallett MB, et al. Transforming Growth Factor-β1 (TGF-β1)-stimulated Fibroblast to Myofibroblast Differentiation Is Mediated by Hyaluronan (HA)-facilitated Epidermal Growth Factor Receptor (EGFR) and CD44 Co-localization in Lipid Rafts. *J Biol Chem*. 2013;288(21):14824. doi:10.1074/JBC.M113.451336
154. Kang KH, Qian ZJ, Ryu BM, Karadeniz F, Kim D, Kim SK. Hepatic Fibrosis Inhibitory Effect of Peptides Isolated from Navicula incerta on TGF-β1

Induced Activation of LX-2 Human Hepatic Stellate Cells. *Prev Nutr Food Sci*. 2013;18(2):124-132. doi:10.3746/PNF.2013.18.2.124

155. Di Sabatino A, Jackson CL, Pickard KM, et al. Transforming growth factor beta signalling and matrix metalloproteinases in the mucosa overlying Crohn's disease strictures. *Gut*. 2009;58(6):777-789. doi:10.1136/GUT.2008.149096
156. Hinz B. Formation and Function of the Myofibroblast during Tissue Repair. *Journal of Investigative Dermatology*. 2007;127(3):526-537. doi:10.1038/SJ.JID.5700613
157. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The Myofibroblast: One Function, Multiple Origins. *Am J Pathol*. 2007;170(6):1807-1816. doi:10.2353/AJPATH.2007.070112
158. Cory G. Scratch-wound assay. *Methods Mol Biol*. 2011;769:25-30. doi:10.1007/978-1-61779-207-6_2
159. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols 2007 2:2*. 2007;2(2):329-333. doi:10.1038/nprot.2007.30
160. Desjardins P, Conklin D. NanoDrop Microvolume Quantitation of Nucleic Acids. *J Vis Exp*. 2010;(45). doi:10.3791/2565
161. Kessler Y, Helfer-Hungerbuehler AK, Cattori V, et al. Quantitative TaqMan® real-time PCR assays for gene expression normalisation in feline tissues. *BMC Mol Biol*. 2009;10:106. doi:10.1186/1471-2199-10-106
162. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3(6):1101-1108. doi:10.1038/NPROT.2008.73
163. Which Cell Culture Media Is Right for You? | Technology Networks. Accessed February 2, 2025. https://www.technologynetworks.com/cell-science/articles/which-cell-culture-media-is-right-for-you-331552
164. Caneparo C, Chabaud S, Fradette J, Bolduc S. Evaluation of a Serum-Free Medium for Human Epithelial and Stromal Cell Culture. *Int J Mol Sci*. 2022;23(17):10035. doi:10.3390/IJMS231710035/S1
165. Yang L, Yan Y. Protein kinases are potential targets to treat inflammatory bowel disease. *World J Gastrointest Pharmacol Ther*. 2014;5(4):209. doi:10.4292/WJGPT.V5.I4.209

166. Zhao Y, Young SL. Requirement of Transforming Growth Factor-β (TGF-β) Type II Receptor for TGF-β-induced Proliferation and Growth Inhibition. *Journal of Biological Chemistry*. 1996;271(5):2369-2372. doi:10.1074/JBC.271.5.2369
167. Cho N, Razipour SE, McCain ML. Featured Article: TGF-β1 dominates extracellular matrix rigidity for inducing differentiation of human cardiac fibroblasts to myofibroblasts. *Exp Biol Med*. 2018;243(7):601. doi:10.1177/1535370218761628
168. Darby IA, Laverdet B, Bonté F, Desmoulière A. Fibroblasts and myofibroblasts in wound healing. *Clin Cosmet Investig Dermatol*. 2014;7:301. doi:10.2147/CCID.S50046
169. Vang Mouritzen M, Jenssen H. Optimized Scratch Assay for In Vitro Testing of Cell Migration with an Automated Optical Camera. *J Vis Exp*. 2018;2018(138):57691. doi:10.3791/57691
170. Sewell GW, Marks DJ, Segal AW. The immunopathogenesis of Crohn's disease: a three-stage model. *Curr Opin Immunol*. 2009;21(5):506. doi:10.1016/J.COI.2009.06.003
171. Boyapati R, Satsangi J, Ho GT. Pathogenesis of Crohn's disease. *F1000Prime Rep*. 2015;7. doi:10.12703/P7-44
172. Halme L, Paavola-Sakki P, Turunen U, Lappalainen M, Färkkilä M, Kontula K. Family and twin studies in inflammatory bowel disease. *World J Gastroenterol*. 2006;12(23):3668-3672. doi:10.3748/WJG.V12.I23.3668
173. Kalla R, Borg-Bartolo SP, Boyapati RK, Satsangi J. Precision medicine in inflammatory bowel disease: concept, progress and challenges. *F1000Res*. 2020;9. doi:10.12688/F1000RESEARCH.20928.1
174. Speca S, Giusti I, Rieder F, Latella G. Cellular and molecular mechanisms of intestinal fibrosis. *World Journal of Gastroenterology : WJG*. 2012;18(28):3635. doi:10.3748/WJG.V18.I28.3635
175. Lucey BP, Nelson-Rees WA, Hutchins GM. HeLa Cells and Cell Culture Contamination-Lucey et al 1463. *Arch Pathol Lab Med*. 2009;133.
176. de Bruyn JR, van den Brink GR, Steenkamer J, et al. Fibrostenotic Phenotype of Myofibroblasts in Crohn's Disease is Dependent on Tissue Stiffness and Reversed by LOX Inhibition. *J Crohns Colitis*. 2018;12(7):849-859. doi:10.1093/ECCO-JCC/JJY036

177. Li C, Grider JR, Murthy KS, et al. Endoplasmic Reticulum Stress in Subepithelial Myofibroblasts Increases the TGF-β1 Activity That Regulates Fibrosis in Crohn's Disease. *Inflamm Bowel Dis*. 2020;26(6):809-819. doi:10.1093/IBD/IZAA015
178. Truffi M, Sorrentino L, Monieri M, et al. Inhibition of Fibroblast Activation Protein Restores a Balanced Extracellular Matrix and Reduces Fibrosis in Crohn's Disease Strictures Ex Vivo. *Inflamm Bowel Dis*. 2018;24(2):332-345. doi:10.1093/IBD/IZX008
179. Romar GA, Kupper TS, Divito SJ. Research Techniques Made Simple: Techniques to Assess Cell Proliferation. *J Invest Dermatol*. 2016;136(1):e1-e7. doi:10.1016/J.JID.2015.11.020
180. Lawrance IC, Rogler G, Bamias G, et al. Cellular and Molecular Mediators of Intestinal Fibrosis. *J Crohns Colitis*. 2017;11(12):1491-1503. doi:10.1016/J.CROHNS.2014.09.008
181. Phan SH. Biology of Fibroblasts and Myofibroblasts. *Proc Am Thorac Soc*. 2008;5(3):334. doi:10.1513/PATS.200708-146DR
182. D'Urso M, Kurniawan NA. Mechanical and Physical Regulation of Fibroblast–Myofibroblast Transition: From Cellular Mechanoresponse to Tissue Pathology. *Front Bioeng Biotechnol*. 2020;8:609653. doi:10.3389/FBIOE.2020.609653
183. Meier JKH, Scharl M, Miller SN, et al. Specific differences in migratory function of myofibroblasts isolated from Crohn's disease fistulae and strictures. *Inflamm Bowel Dis*. 2011;17(1):202-212. doi:10.1002/IBD.21344
184. Shahini A, Shahini A. Role of interleukin-6-mediated inflammation in the pathogenesis of inflammatory bowel disease: focus on the available therapeutic approaches and gut microbiome. *J Cell Commun Signal*. 2023;17(1):55. doi:10.1007/S12079-022-00695-X
185. Noble AJ, Nowak JK, Adams AT, Uhlig HH, Satsangi J. Defining Interactions Between the Genome, Epigenome, and the Environment in Inflammatory Bowel Disease: Progress and Prospects. *Gastroenterology*. 2023;165(1):44-60.e2. doi:10.1053/J.GASTRO.2023.03.238
186. Humphreys DT, Lewis A, Pan-Castillo B, et al. Single cell sequencing data identify distinct B cell and fibroblast populations in stricturing Crohn's disease. *J Cell Mol Med*. 2024;28(9). doi:10.1111/JCMM.18344

187. Mukherjee PK, Nguyen QT, Li J, et al. Stricturing Crohn's Disease Single-Cell RNA Sequencing Reveals Fibroblast Heterogeneity and Intercellular Interactions. *Gastroenterology*. 2023;165(5):1180-1196. doi:10.1053/j.gastro.2023.07.014
188. Szklarczyk D, Kirsch R, Koutrouli M, et al. The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res*. 2023;51(D1):D638-D646. doi:10.1093/NAR/GKAC1000
189. Harper JM. Primary Cell Culture as a Model System for Evolutionary Molecular Physiology. *Int J Mol Sci*. 2024;25(14):7905. doi:10.3390/IJMS25147905
190. Piwocka O, Musielak M, Ampuła K, et al. Navigating challenges: optimising methods for primary cell culture isolation. *Cancer Cell Int*. 2024;24(1):1-12. doi:10.1186/S12935-023-03190-4/FIGURES/6