New Insights into the Treatment and Pathophysiology of Fibrostenotic Crohn's Disease

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Thesis submitted in fulfilment of the requirements for the degree of
Doctor in Medical Sciences
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Funding

This work was funded by the Flemish Agency for Innovation by Science and Technology (IWT; project number IWT120514) and the Research Foundation Flanders (FWO; 11S7416N).

Please refer to this work as:

Holvoet, T. (2018) New Insights into the Treatment and Pathophysiology of Fibrostenotic Crohn's Disease. PhD thesis, Ghent University, Belgium.

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List of Abbrevations

ASUC	Acute Severe Ulcerative Colitis
ATG16L1	Autophagy-Related 16 Like-1
ATG	Autophagy-Related protein
ATM	Adipose tissue-associated macrophages
ATT	Adipose tissue-associated T cell lymphocytes
CARD15	Caspase recruitment domain containing proteins 15
CD	Crohn's Disease
CRC	Colorectal carcinoma
CTGF	Connective tissue growth factor
DSS	Dextran sodium sulphate
EGF	Epithelial Growth Factor
EndoMT	Endothelial-to-mesenchymal transition
EMT	Epithelial-to-mesenchymal transition
EWAS	Epigenome-wide association study
GWAS	Genome-wide association study
HLA	Human Leukocyte Antigen
IBD	Inflammatory Bowel Diseases
ICC	Interstitial cell of Cajal
IEC	Intestinal Epithelial Cell
IFN	Interferon
IL	Interleukin
JAK	Janus Kinase
LRR	Leucine-rich repeating region
MAGI1	Membrane-associated guanylate kinase WW and PDZ domain-containing protein 1
MAT	Mesenterial adipose tissue
MDP	Muramyl dipeptide
MHC	Major Histocompatibility Complex
miR	microRNA
MMP	Matrix Metalloproteinases
MRTF	Myocardin related transcription factor
NFκB	Nuclear Factor kappa B
NK	Natural Killer cells
NOD2	Nucleotide-binding oligomerization domain-containing 2
PBMC	Peripheral Blood Mononuclear Cells
PDGF	Platelet Derived Growth Factor
PPAR	Peroxisome proliferator activated receptor
PTEN	Phosphatase and tensin homologue
RAAS	Renin Angiotensin Aldosteron System
SC	Stellate Cell
SEMF	Subepithelial myofibroblasts
SMA	Smooth muscle actin
SMAD	Signal transducer and activator of transcription
SMC	Smooth Muscle Cell
SNP	Single Nucleotide Polymorphism
SPRY-1	Sprouty-homolog 1

SRF	Serum Response Factor
TGFβ	Transforming Growth Factor beta
TLR	Toll-like receptor
TNFα	Tumor Necrosis Factor alpha
UC	Ulcerative colitis

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Summary

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract with two distinct clinical entities: Crohn's disease (CD) and ulcerative colitis (UC). In approximately one third of patients, especially in CD but to a lesser extent also in UC, these repeating cycles of inflammation will lead to the accumulation of scar tissue (fibrosis), resulting in narrowing of the intestinal lumen, stenosis and gastro-intestinal obstruction. The pathophysiology of intestinal fibrosis is currently incompletely understood and no specific anti-fibrotic therapies are available, leaving surgery and its associated loss of viable intestinal tissue as the only therapeutic option.

In the first chapter of this thesis (**Chapter III.1**), local Rho kinase inhibition was identified as an attractive new treatment strategy for fibrostenotic CD. Rho kinases are small serine/threonine kinases involved in cytoskeletal organization and central in several aspects of fibrogenesis making them attractive targets for anti-fibrotic therapy although systemic side-effects such as cardiovascular hypotension limit their applicability in other organ systems. In the first part of the chapter, we showed Rho kinases to be involved in CD-related fibrosis with enhanced enzyme activity in ileal biopsies from both active CD and fibrostenotic segments compared to normal ileal tissue. Next, a locally active Rho kinase inhibitor (AMA0825) was developed, bypassing the problems associated with systemic Rho kinase inhibition. In a series of murine experiments, AMA0825 was then shown to effectively prevent intestinal fibrosis, both in monotherapy and in combination with anti-tumor necrosis factor (TNF) therapy. Additionally, the compound was shown to regress already established fibrosis which is clinically important as approximately 10% of patients already has fibrostenotic complications at the time of CD diagnosis. Mechanistically, Rho kinase inhibition was shown to affect multiple pathways involved in fibrogenesis including activation of intestinal fibroblasts and smooth muscle cells (both key cells in the pathophysiology of fibrosis), epithelial-to-mesenchymal transition (EMT) (a transformational process increasing the number of extracellular matrix producing fibroblasts through transformation of epithelial and/or endothelial cells), and by interfering with the normal autophagy pathways in fibrosis. In a last set of translational experiments, AMA0825 was shown to reduce profibrotic secretion of ileal samples taken from patients with fibrostenotic CD suggesting strongly that the anti-fibrotic effects are transferrable to the human situation.

However, constructing clinical trials to evaluate anti-fibrotic therapy is difficult due to problems with patient selection as there is currently no good way to predict which CD patients will develop fibrostenosis. A better understanding of the genetics of early fibrostenotic CD might help solve this

problem and was explored in **Chapter III.2** of this thesis. In a multi-centric, retrospective genetic association study using a well-phenotyped population of fibrostenotic CD based on computed tomography/magnetic resonance imaging, genetic risk factors for early fibrostenotic CD were investigated. Several association with single nucleotide polymorphisms or SNPs were found, not only suggesting a genetic component to early fibrostenotic CD but also linking previously unrelated disease pathways to intestinal fibrosis, including *MIS18BP1* (rs35223850) involved in centromere assembly and oxidative stress protection (gluthation-peroxidase 4 (*GPX4*-rs17554931)). Other identified SNPs could be linked directly to existing pathways such as EMT (cadherin-4, *CHD4*, rs4925207) or indirectly through proximity to interesting candidate genes (e.g. *TNFAIP3* and the rs113661016 SNP). Interestingly, carrying three or more of these risk variants greatly shortened the time it took to develop the fibrostenotic complication, strongly suggesting an important role for genetics in the pathophysiology of early fibrostenotic CD.

Another problem arising when constructing clinical trials to evaluate anti-fibrotic therapies in CD, aside from patient selection, is the follow-up of therapeutic effect. Biomarkers could provide a solution to this and were the subject of the third chapter of this thesis (**Chapter III.3**). In an exploratory study, combining serum levels of matrix metalloproteinase -2 and -3 (MMP-2 and -3) with tissue inhibitor of MMP-3 (TIMP-3) proved to be discriminative for fibrostenotic CD, at least in patients with evidence for active inflammation. Moreover, low serum levels of MMP-10 appeared to be predictive of fibrostenosis development long before these complications arose. Although this was only a preliminary study and results should be confirmed in prospective trials, these findings are promising towards finding a clinically usable biomarker for fibrostenotic CD.

Lastly, another controversial issue in the management of patients with IBD was targeted. Using corticosteroids for the induction of remission in both CD and UC is common practice but based on very limited data dating back from the 1950s. In the last chapter of this thesis (**Chapter III.4**) a systematic review and meta-analysis according to the Cochrane methodology was performed exploring the evidence of using corticosteroids for induction of remission in UC. Oral corticosteroids were found to lead to significantly greater clinical, endoscopic and histological remission compared to placebo without inducing significantly more adverse events or withdrawals due to adverse events. Additionally, locally active corticosteroids were also able to induce clinical, but appeared less effective in inducing endoscopic remission compared to systemic corticosteroids, although they were associated with less frequent adverse events. This study provided sound evidence for the use of oral corticosteroids (both systemic and locally active) in the treatment of UC.

Samenvatting

Inflammatoire darmziekten (IBD) worden gekenmerkt door chronische, recurrente ontsteking van de dikke en dunne darm. Hierbij worden twee verschillende klinische entiteiten onderscheiden: de ziekte van Crohn (CD) en colitis ulcerosa (UC). Bij ongeveer een derde van de patiënten, frequenter voorkomend in CD dan in UC, leiden deze zich herhalende onstekingscyclussen tot het ontstaan van littekenweefsel in de darm (fibrose) met progressieve vernauwing van het darmlumen met uiteindelijk stenoses en darmobstructies tot gevolg. Het exacte ontstaansmechanisme van deze darmfibrose is tot op heden onvolledig gekend noch zijn er specifieke behandelingen beschikbaar, zodat chirurgie met verwijderen van vitale delen van de darm als enige therapeutische optie overblijft.

In het eerste hoofdstuk van deze thesis (**Hoofdstuk III.1**) werd bewijs aangebracht voor locale rho kinase inhibitie als therapie voor stenoserende vormen van de ziekte van Crohn. Rho kinase zijn kleine serine/threonine kinases betrokken in de cytoskeletorganisatie die een centrale rol spelen in verschillende processen betrokken in fibrose en hen tot aantrekkelijke doelwitten maakt voor anti-fibrotische therapie. Systemische bijwerkingen zoals cardiovasculaire hypotensie limiteren hun toepasbaarheid echter in belangrijke mate. In het eerste deel van dit hoofdstuk werd aangetoond dat Rho kinases een belangrijke rol spelen in ziekte van Crohn-gerelateerde fibrose met toegenomen enzymactiviteit in actief geïnflammeerde en gestenoseerde ileale segmenten vergeleken met normaal ileum. Vervolgens werd een lokaal actieve Rho kinase inhibitor (AMA0825) ontwikkeld om de bijwerkingen van systemische Rho kinase inhibtie te ontwijken. In verschillende muismodellen werd vervolgens aangetoond dat toediening van AMA0825 het ontstaan van intestinale fibrose kon tegengaan en dit zowel in monotherapie als in combinatie met anti-tumor necrosis factor (TNF) therapie. In bijkomende experimenten werd aangetoond dat de behandeling eveneens reeds langbestaande fibrose kan omdraaien, een belangrijk gegeven aangezien meer dan 10% van de patiënten reeds fibrostenotische complicaties heeft op het moment van hun diagnose. Op mechanistisch vlak interfereert Rho kinase inhibitie met verschillende actiemechanismen betrokken in de pathofysiologie van fibrosis waaronder activatie van fibroblasten en gladde spiercellen, epithiliale-naar-mesenchymale transitie (EMT) (waarbij epitheelcellen en/of endotheelcellen omgezet worden naar fibroblasten) en eveneens met de normale autofagie respons tijdens fibrogenese. In een set van translationele experimenten verminderde AMA0825 de secretie van profibrotische cytokines uit ileale biopten verzameld uit patiënten met fibrostenotische Crohn, wat suggereert dat deze resultaten extrapoleerbaar zijn naar de humane situatie.

Het opbouwen van een klinische studie om anti-fibrotische therapie te testen is echter geen gemakkelijke opgave. Er zijn immers geen manieren om accuraat te voorspellen welke CD patiënten precies fibrotische complicaties zullen ontwikkelen. Een beter inzicht in de genetica van deze aandoening kan mogelijks een oplossing bieden. In **Hoofdstuk III.2** van deze thesis werd in een multi-centrische, retrospectieve genetische associatiestudie gezocht naar genetische factoren betrokken in het vroegtijdig ontstaan van fibrostenotische complicaties. Patiënten in deze studie werden grondig geselecteerd op basis van computed tomography (CT) of magnetic resonance imaging (MRI) beelden en verschillende single nuleoctide polymorphisms of SNPs werden hierbij geïdentificeerd. De resultaten van deze studie suggereren niet alleen dat er een belangrijke genetische bijdrage is tot het vroegtijdig ontwikkelen van fibrotische complicaties in CD, maar tonen ook de betrokkenheid van nieuwe, voordien niet gekende ziektemechanismen in de pathofysiologie van fibrose. Zo is dit de eerste studie die de link legt tussen fibrose en bijvoorbeeld centromere opbouw (via *MIS18BP1* (rs35223850)) en oxidatieve stress (gluthation-peroxidase 4 (*GPX4*-rs17554931)). Andere gevonden associaties konden dan weer direct gelinkt worden aan gekende ziektemechanismen zoals EMT (cadherin-4, *CHD4*, rs4925207) of soms indirect via nabijheid van andere kandidaatgenen (e.g. *TNFAIP3* and the rs113661016 SNP). Een interessante bevinding van deze studie is dat patiënten die meerdere van deze risk varianten dragen, significant sneller fibrose ontwikkelden dan diegene die geen drager waren van deze varianten. Dit suggereert nog maar een belangrijke rol voor de genetica in de pathofysiologie van zich vroeg ontwikkelende fibrostenotische CD.

Naast patiëntenselectie, zijn manieren om patiënten te volgen een groot probleem tijdens klinische studies rond anti-fibrotische therapie in CD. Biomerkers zouden mogelijks een oplossing kunnen bieden en vormden het onderwerp van het derde hoofdstuk van deze thesis (**Hoofdstuk III.3**). Een exploratieve studie toonde dat combineren van de serumwaarden van matrix metalloproteinases-2 and -3 (MMP-2 en -3) samen met tissue inhibitor of MMP-3 (TIMP-3) kan discrimineren tussen fibrostenotische en ongecompliceerde CD, tenminste in patiënten met aanwezigheid van inflammatie. Bovendien bleken lage serum concentraties van MMP-10 predictief voor fibrose ontwikkeling lang voor het verschijnen van deze complicaties. Alhoewel dit bevestigd moet worden in prospectieve studies, zijn deze resultaten alvast veelbelovend in functie van het vinden van een geschikte biomerker voor fibrostenose in CD.

In het laatste hoofdstuk (Hoofdstuk III.4) wordt ingegaan op een ander controversieel thema binnen de behandeling van IBD. Corticosteroïden worden daglelijks gebruikt voor het induceren van ziekteremissie in zowel CD als UC, maar de evidentie hiervoor is beperkt en vooral gebaseerd op data

uit de Jaren ’50. In dit hoofdstuk wordt de wetenschappelijke evidentie voor dit gebruik nagegaan via een systematische review en meta-analyse gebruikmakend van de Cochrane methodologie. Op basis van alle studies die in de afgelopen jaren over dit onderwerp verschenen zijn, blijken orale corticosteroiden superieur in het inducer van zowel klinische, endoscopische als histologische remissie ten opzichte van placebo en dit zonder significant meer bijwerkingen te veroorzaken. Lokaal werkende preparaten bleken even effectief in het induceren van klinische remissie en dit met minder bijwerkingen, maar bleken minder efficient in het bereiken van endoscopische remissie. Samengenomen toonde deze studie dat er voldoende bewijs is om corticosteroiden te gebruiken om remissie te induceren in patiënten met UC.

CHAPTER I

INTRODUCTION

I. INFLAMMATORY BOWEL DISEASE

Inflammatory bowel diseases (IBD) are a group of idiopathic inflammatory disorders affecting the small bowel and colon, characterized by a chronic relapsing-remitting disease course and comprising two distinct clinical entities: Crohn's disease (CD) and ulcerative colitis (UC).[1]

Discovered by dr. Crohn and dr. Oppenheimer in 1932, IBD affects about 1 in 200 persons with highest prevalence rates in Northern Europe and North America while being significantly less frequent in developing countries. However, incidence rates are on the rise, especially in the developing world.[2] Annual incidence rates in Western Europe are estimated between 0.5 to 10.6 and 0.9 to 24.3 cases per 100.000 persons for CD and UC respectively.[3] Aside from a clear geographical distribution, incidence rates also diverge between ethnic groups, with certain Jewish populations having a particularly high risk, while Asian, Hispanic, black people and American Indians appear to be less frequently affected compared to the Caucasian population.[2,4]

Diagnosis of IBD is based on a combination of clinical, endoscopic and histological findings.[1] Typically patients will present with symptoms of chronic (sometimes bloody) diarrhoea, abdominal pain, cramps, weight loss and/or fever. Extra-intestinal manifestations are common, affect about 20-40% of patients and can target the joints (ranging from peripheral arthritis to spondylarthropathy), the liver (primarily primary sclerosing cholangitis), eyes (e.g. episcleritis and scleritis) and the skin (e.g. pyoderma gangrenosum).[5]

Although CD and UC are generally considered twin disorders and share many clinical and pathophysiological features, important differences exist. Inflammation in CD is discontinuous, transmural and can affect all areas of the gastro-intestinal system (from mouth to anus but favouring the terminal ileum), while UC is characterized by a more superficial inflammatory response generally limited to the colon. Pathophysiologicaly this is reflected by differences in the type of inflammatory response underlying both diseases: where T helper (Th) 1 and Th17 responses are predominant in CD with subsequently high levels of interferon (IFN) γ, tumor necrosis factor (TNF) α and interleukin (IL) 17, a shift towards a Th2 response and resulting high levels of IL5, IL13 and TNFα is seen in UC.[6,7] The distinction between both disease phenotypes can be readily made on endoscopy: diseased regions of the gut separated by normal appearing mucosa (so-called skip lesions), as well as deep ulcerations, "cobblestones" and rectal sparing are typically seen in CD. In UC, on the other hand, inflammation is continuous starting from the rectum with superficial erosions and friability of the mucosa.

Additionally, distinctive histopathological features can be helpful in making the differential diagnosis between UC and CD.[8] Aside from differences in the depth and continuity of inflammation, CD is typically characterized by the presence of epithelioid granulomas, mucin preservation and focal inflammation by plasma- and lymphocytes, while in UC mucin depletion, diffuse infiltration by neutrophils, eosinophils and plasma cells, and crypt distortion are the most discriminative features.[9]
In this section, etiology and risk factors for IBD will be discussed along with the currently available therapeutic options and frequently occurring complications of the disease.

1. IBD as a multifactorial disease

Although the discovery of IBD dates back almost one hundred of years, what exactly causes the disease is not fully understood. The current accepted paradigm states that IBD results from a disturbed interaction between the environment and the microbiota which leads to an exaggerated immune response in genetically susceptible persons.[8]

Evidence for a genetic component to IBD is well-established and was first hinted upon in family aggregation and twin studies with 15% of IBD patients having a first degree relative affected by the disease and concordance rates of 20-50 % in monozygotic twins and 10% in dizygotic twins.[10,11] In UC, twin concordance rates are lower reaching up to 16% and 4% in mono- and dizygotic twins respectively.[12] Genome-wide associations studies (GWAS) have been able to identify over 242 risk loci for IBD, which will be discussed in more detail in section 3 of this thesis.[13] Although enormous progress has been made in clarifying the genetic risk in IBD, all of the risk loci identified today only explain, respectively, 13% and 9% of the disease variance in CD and UC, underscoring the importance of both external and currently unknown heritable factors ("missing heritability") in disease pathology.[14]

Environmental factors, collectively known as the "**exposososome**", are believed to play an important role in IBD and are closely connected to the Western lifestyle.[15] Rising incidence rates in Europe and North America since the second half of the 20th century, together with a clear geographical distribution with an underrepresentation of IBD in developing countries, pointed clearly towards an important contribution of lifestyle factors.[2] Since then, an array of environmental factors has been shown to contribute to the onset of IBD or to induce flare-ups of the disease. Examples include dietary factors (e.g. vitamin D deficiency), food additives (most frequently aluminium or TiO2), air and water pollution, medication use (e.g. non-steroidal anti-inflammatory drugs, oral anticonceptives), stress and other lifestyle factors such as smoking.[15] One popular hypothesis (the so-called "hygiene hypothesis") states that the rising incidence in IBD seen with modernisation is partly attributable to a lack of exposure to infectious agents in early childhood which impairs immune tolerance to the

microbiota.[16] Unfortunately, many of the epidemiological studies performed in IBD yield conflicting results, making it difficult to derive definite relationships between environmental factors and disease development.

Disturbances in the gut microbiota (or dysbiosis) have been extensively linked to IBD. Strong evidence comes from murine experiments in which germ-free animals show reduced or even absent inflammation in several models of experimental ileitis (e.g. TNF $^{\Delta are}$ model) and colitis (e.g. IL10$^{-/-}$ knockout mice), however not in dextran sodium sulphate (DSS)-induced colitis.[17] In human IBD patients, it has been long known that diversion of the faecal stream (and thus the big bulk of microbiota) can induce remission in refractory CD patients and relapse occurs in the majority of patients when the stoma is removed.[18] Additionally, antibiotics have been shown to adequately prevent post-surgical recurrence in IBD and are frequently used in the treatment of perianal disease although they fail to induce remission in other instances.[8,19] Although several bacteria have been linked to IBD, no single causative organism has been identified. Instead there seems to be a disturbance in the global microbial composition most typically an increase in the *Enterobacteriaceae* (phylum *Proteobacteria*), a reduced number of *Firmicutes* (especially *Clostridium Leptum*) and overall a diminished microbial diversity.[20] *Bacteroidetes* levels seem to differ as well between IBD patients and healthy controls but data are more ambiguous. The same dysbiotic profiles seem to be present in the mucosa of IBD patients with most strikingly a decrease in *Clostridium clusters XIVa and IV*.[21] Depletion of the butyrate-producing *Roseburia hominis* and *Faecalibacterium prausnitzii* are of particular interest as butyrate has known anti-inflammatory properties. Additionally, low levels of F. Prausnitzii in the ileal mucosa has been shown to be associated with a higher risk of postoperative recurrence.[22] As these dysbiotic changes are also present in patients in remission and even in relatives of IBD patients, the exact causal relationship with disease initiation remains unclear.[23,24]

Aside from environmental factors, a dysregulation of both the innate and adaptive immune system resulting in a persistence of the inflammatory response is seen in IBD. The **innate immune system** normally represents the first line of defence of the gut against micro-organisms and consists of the epithelial barrier and innate immune cells such as macrophages and dendritic cells (DCs).[25] The integrity of the **epithelial barrier** is maintained through the tight junctions, adherens junctions and desmosomes that tightly link the intestinal epithelium together but this integrity has been shown to be compromised in IBD allowing for bacterial translocation and stimulation of the immune system. Recent evidence from GWAS studies linking polymorphisms in e.g. CDH1 to IBD, a gene that encodes for E-cadherin (a main component of adherens junctions), suggest that this is not a mere byproduct

of intestinal inflammation a primary disease mechanism.[26] **Macrophages** form a heterogenous population within the gut mucosa and are responsible for bacterial phagocytosis and recruitment of other immune cells. In IBD, however, an increased proportion of macrophages have been found to express CD14, a co-receptor for lipopolysaccharide (LPS) signalling (a component of the bacterial cell wall) leading to an exaggerated production of inflammatory mediators upon stimulation and propagation of the inflammatory response.[27] **DCs** on the other hand are antigen-presenting cells that form a link between the innate and adaptive immune systems. In IBD, higher levels of activated DCs (expressing CD40) have been found in the lamina propria. Additionally, DCs form IBD patients secrete higher amounts of TNF and IL8 upon stimulation with LPS.[28]

In contrast, the adaptive immune system represents a highly specific immune response in which **T cells** are the key cells. Especially in CD, there appears to be an exaggerated IFNγ-producing **Th1** response, while **Th2 cells** (producing IL4, IL5 and IL13) have historically been associated with UC.[26] Indeed, activated T cells from the mucosa of CD patients produce higher amounts of IFNγ compared to UC patients, while the latter produce more IL5.[26] Besides Th1 and Th2 cytokines, there is an increased expression of IL-17 in both serum and inflamed mucosa of IBD patients, indicating an important role of the **Th17 subtype** in IBD pathogenesis.[29] Lastly, regulatory T cells (**Tregs**) are characterised by Foxp3 expression and are able to inhibit Th0 cell proliferation (the precursor cell for Th1 and Th2 cells) thereby suppressing abnormal immune responses to gut antigens. Treg levels are lower in the serum of patients with active IBD compared to quiescent patients.[30] Moreover in murine models, Tregs have important anti-inflammatory effects. [31]

2. Therapeutic approaches

Current therapeutic goals in IBD have changed from mere symptomatic control (clinical remission) to achieving complete mucosal healing or endoscopic remission. For achieving this a step-up approach is used, in which the most effective (and expensive) therapeutic options are reserved for refractory patients. In this view surgery is typically regarded as a bail-out option for patients refractory to medical therapy. Several studies have investigated the reverse strategy (top-down approach with an early introduction of biological therapy and surgery) and although remission rates achieved were better they did not justify the cost increase.[8,32]

Immunomodulators like 5-aminosalicylates (5-ASA) are considered first-line therapy for UC and are sufficient in over 50% of UC patients but are generally ineffective in CD. First-line therapy for CD typically consists of corticosteroids, which have a broad immunosuppressive effect by reducing T- and

B cell mediated inflammatory cytokines release. In UC, they are considered second-line therapy (to 5-ASA) but still hold an important place in the management of the disease (as recently confirmed by our Cochrane analysis (see Chapter III.4)). In case of treatment failure or corticosteroid dependence, immunosuppressive agents such as azathioprine or methotrexate are added, followed by introduction of biological therapy if necessary.[8,32]

Biological therapy consists of antibodies directed against certain key proteins in the pathophysiology of IBD and one of the best known classes are directed against TNF (**TNF antagonists**). Since their first introduction 20 years ago, they have become the cornerstone of refractory IBD management. Several products (e.g. infliximab, adalimumab, golimumab) are available which differ in the way they are administrated but are generally considered to be equally effective in inducing remission.[8,32] In 2015, a new type of biological entered the market targeting $\alpha 4\beta 7$ integrin (**vedolizumab**) and interfering with leukocyte trafficking to the gut.[33,34] More recently, biological therapy directed against the IL12/IL23 pathway (**ustekinumab**) became available for patients with CD.[35]

Several new medications targeting completely different pathways are currently in clinical trial and include **Janus kinase (JAK) inhibitors**, directed against a family of signal transducing proteins involved in cytokine signalling. Tofacitinib (a JAK1/3 inhibitor) has shown promising effects in the treatment of UC, while a recent phase II trial shows efficacy of filgotinib, a selective JAK1 inhibitor, in CD.[36,37] **Mongersen**, a SMAD7 antisense oligonucleotide, enhances the anti-inflammatory TGFβ pathway and achieves remarkable CD remission rates in a phase II trial (although preliminary results from the phase III trial are negative), while modulators of the sphingosine-1-phosphate-1 receptor ($S1P_{1R}$ modulators, ozanimod) sequestering the lymphocytes within the lymph nodes holds promise as well.[37,38]

3. Natural disease course and complications

3.1. Natural disease course of UC

At diagnosis, about one third of UC patients will present with rectal disease, roughly one third with left-sided colitis and similar numbers will have pancolitis at diagnosis. With time, disease extent seems to progress with about 15-30% of patients with initial left-sided colitis that will present with pancolitis at some point in their disease course.[39]

From long-term follow-up studies like the IBSEN cohort, we know that the disease tends to be most aggressive in its early years, with the majority of colectomies being performed in the first years after

diagnosis. In total, about 10% of patients will require colectomy because of acute severe colitis (ASUC) refractory to medical therapy.[39,40]

Colorectal cancer (CRC) has been a major concern in long-standing IBD, especially in UC patients. The relative risk to develop CRC is approximately doubled in UC patients as compared to the general population, however absolute risk in general remains quite low between 1.1 and 5.3% after 20 years of disease duration.[40] Early diagnosis, concomitant primary sclerosing cholangitis, extensive disease and long disease duration are increase CRC risk in an important way.[41]

3.2. Natural disease course of CD

In CD, disease location (categorized by the Montreal classification[42]) is generally more stable over time as compared to UC: at diagnosis about 40% of patients will have colonic disease (Montreal L2), 30% ileocolonic (Montreal L3) and in 30% only the small bowel (Montreal L1) will be affected.[39] Four percent of patients will have involvement of the upper gastrointestinal tract (Montreal L4). These numbers tend to stay stable over the course of the disease with only 14% begin reclassified after 5 years of follow up in the IBSEN cohort.[43]

Disease complications occur more frequently in CD compared to UC. Approximately 19% of patients already show evidence of an intestinal complication at diagnosis and eventually about two thirds of all CD patients will develop a complicated disease course within 20 years of the diagnosis.[44] About half of these patients presents with a penetrating disease phenotype (classified as Montreal B(behaviour)3) which is characterized by the development of fistulas or "tracts" between two epithelium-lined surfaces.[45] Fistulas are most frequently seen in the perianal region (54%), are entero-enteric (24%) or recto-vaginal (9%) and development seems to be closely linked to colorectal disease involvement.[46] Why certain patients develop penetrating disease while others do not, is incompletely understood. However, there appears to be a genetic component with several susceptibility genes identified over these past years. For instance, in the European IBDchip project, the carriage of the rs7746082 variant in the PR domain containing 1 with ZNF domain gene (*PRDM1*) or any Nucleotide-binding oligomerization domain-containing 2 (*NOD2*) variant was strongly associated with an increased risk for developing penetrating disease, while the rs11465804 variant in the Interleukin 23 receptor (*IL23R*) gene appeared to be protective.[45,47] As antibiotics are routinuously and successfully used in the treatment of fistulising disease, microbiota are believed to play an important role.[45] However, no study to date has been able to isolate or culture a specific set of microbiota from fistulising tracts which suggest that a permanent infection is not at the heart of the problem.

Peptidoglycans, components of the bacterial cell wall, however, are abundantly present and might contribute to the ongoing inflammation seen in these fistulas.[48,49]

Intestinal fibrosis resulting in intestinal stenosis and strictures (Montreal B2) is another frequent complication of CD and affects the other half of CD patients with a complicated disease course. Its pathophysiology and management will be discussed in more detail in section II of this thesis. Patients who do not develop these complications are considered to have a purely inflammatory disease and are classified as B1 in the Montreal classifcation.[44]

II. FIBROSTENOTIC CROHN'S DISEASE

Intestinal fibrosis results from the excessive accumulation of extracellular matrix (ECM) proteins by activated mesenchymal cells and is induced by repeated cycles of inflammation. This accumulation leads to a progressive thickening of the bowel wall and subsequently narrows the intestinal lumen leading to the formation of stenosis, strictures and a fibrostenotic phenotype. Until recently, intestinal fibrosis was regarded as an irreversible consequence of repeated cycles of inflammation, however accumulating evidence has put a new perspective to this vision. In this section, an overview of the epidemiology, pathophysiology, diagnosis and currently available treatment modalities for intestinal fibrosis will be given.

1. Epidemiology, natural history and risk factors for fibrostenotic Crohn's disease

Although most CD patients present with a purely inflammatory disease at diagnosis, approximately 10% will already have fibrostenotic complications at this point.[50] Ten years onwards, 30% of patients referred to tertiary centres will have evolved to a fibrostenotic disease phenotype.[51] The prevalence in population-based studies is generally lower, but even there more than 20% of CD patients develop stenosis within the first 20 years of their disease.[44]

Fibrostenosing disease is one of the main indications for surgery in CD and results in a cumulative risk of resection between 40 and 71% within 10 years of diagnosis.[44] Surgery is often the only available treatment option for these patients: current medical therapy for intestinal fibrosis is lacking and despite the recent advances in anti-inflammatory treatment in IBD, incidence of fibrostenotic disease has not changed over the last 20 years.[50,52]

Fibrostenosis can develop in every CD affected bowel segment (including the upper gastro-intestinal system), but seems to follow the segmental distribution of inflammation. Strictures are therefore most frequently seen in the ileocaecal region and terminal ileum, presumably also in part because of the smaller diameter of the ileum compared to the colon leading to fibrostenosis becoming more rapidly relevant.[52] Additionally, recurrence at the site of anastomosis after bowel resection is also very common with about 70% of patients requiring additional surgery.[50]

Risk factors for fibrostenotic disease have been identified but show an important overlap with complicated disease in general (e.g. fibrostenotic or fistulising disease, need for surgery) making them at best only partly related to fibrostenosis.[52] Age below 40 years at diagnosis, concomitant perianal

disease and the need for corticosteroids during the first disease flare all have been associated with fibrostenotic disease, but are general predictors of a complicated disease course. If the patient exhibits two or more of these factors, chances of a debilitating course are more than 90%.[53] Similarly, smoking is a risk factor associated with complicated CD. The same type of bias applies to the currently available biomarkers, which will be discussed in more detail in section II.5.

Although typically seen as two different CD phenotypes there is an important association between fibrostenosing and fistulising disease with the presence of penetrating disease often accompanying strictures. The presence of fistulas holds a positive predictive value for concomitant stenosis (86,2%), and some experts believe that strictures precede fistulisation in the progression of complicated CD. No prospective studies, however, have been performed to support this hypothesis.[50,52,54]

Fibrostenotic complications have primarily been linked to CD, but recent evidence shows that fibrosis is also present in UC. The prevalence of fibrotic strictures in UC ranges from 2 to 11% compared to 8% in colonic CD. Moreover, in 100% of colectomy specimens from UC patients some degree of fibrosis is found on histology, even in the absence of a clear stricture. Although fibrosis in UC is generally more superficial than in CD (in accordance with the depth of inflammation), it has functional consequences and can affect colon motility in an important way.[50,52,55,56]

2. Pathophysiology of intestinal fibrosis

Fibrogenesis in itself is a physiological process involved in tissue repair and wound healing. Triggered by inflammation or other disease-related injuries, it has adaptive features in the short run but when progressing over a prolonged period of time it will lead to parenchymal scarring, cellular dysfunction and ultimately organ failure.[57] Four major phases are typically seen in fibrogenesis: first a primary injury (often an inflammatory trigger) to the tissue initiates the fibrotic response. Effector cells (e.g. fibroblasts and myofibroblasts, muscle cells) are activated in the second phase and will lead to of the synthesis and secretion of extracellular matrix components. In the final phase, which coincides with the previous phases, fibrogenesis becomes auto-propagatory; ECM expansion becomes independent of the initiating injury and progression to organ failure will follow.[50,57]

Fibrosis is a common pathway leading to organ dysfunction in several organ systems. Cardiac fibrosis results from ischaemic injury to the heart, leads to disturbances in both systolic and diastolic function and predisposes the heart for arrythmias.[58] In the liver, fibrosis is a well-known complication of viral hepatitis, auto-immune or metabolic diseases and when left unchecked leads to liver cirrhosis and

portal hypertension.[59] Diabetes and hypertension are the most important causes of renal fibrosis and eventually result in chronic kidney failure requiring renal replacement therapy.[60] Pulmonary fibrosis can be initiated by repeated infections or silica exposure but is in most cases idiopathic (IPF) and is characterized by parenchymal honeycombing, reduction in lung compliance and restriction of the lung function.[61] Lastly, fibrosis of the skin is commonly seen in systemic sclerosis and in hypertrophic scar tissue.[62] In all of these organs, fibrosis is well studied and therapeutic options are even available in some instances (e.g. ACE inhibitors in cardiac fibrosis, pirfinidone in lung fibrosis, cfr infra). Intestinal fibrosis, in comparison, is relatively unknown and understudied. However, some of the basic principles discovered in other organ systems are applicable to the gut as well. In this chapter an overview of the different pathophysiological mechanisms involved in intestinal fibrosis are given (**Figure 1**).

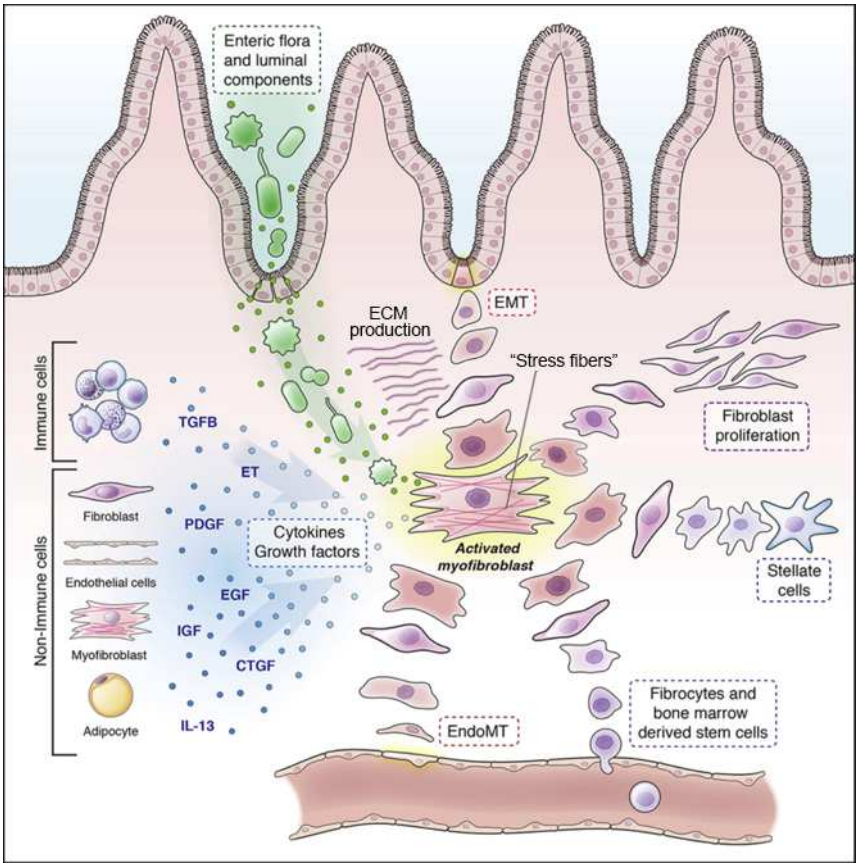

Figure 1 – Overview of the pathophysiology of intestinal fibrosis. The myofibroblast is the central effector cell in intestinal fibrosis and is stimulated by several soluble mediators and bacteria. Myofibroblasts originate from numerous cell types, are characterized by the appearance of so called 'stress fibers' and are responsible for ECM production. TGFβ = transforming growth factor, PDGF = platelet derived growth factor, CTGF = connective tissue growth factor, EGF = epithelial growth factor, IGF = insulin-like growth factor, IL = interleukin, ET = endothelin, EMT = epithelial-to-mesenchymal transition, EndoMT = endothelial-to-mesenchymal transition. Adapted from Rieder et al.[50]

2.1. Cell types involved in fibrosis

Damage to the intestinal tissues, caused by for example inflammation but also by ischemia or chemical agents, will trigger injured epithelial and endothelial cells to release inflammatory cytokines and chemotactic factors promoting recruitment and activation of a wide range of immune cells of both the adaptive and innate immune system (including macrophages, lymphocytes, polymorphonuclear leukocytes, eosinophils, basophils and mast cells).[57] Together they will activate various effectors cells that drive the fibrogenetic process. In this chapter an overview of the different cell types involved in intestinal fibrogenesis will be given.

2.1.1. Myofibroblasts

Activated myofibroblasts are believed to be the key effector cells in intestinal fibrosis, actively secreting ECM proteins and promoting a cytokine environment which supports and maintains the fibrotic process.[63]

Myofibroblasts are highly contractible cells expressing typical cellular markers as vimentin (a type III intermediate filament), α-smooth muscle actin, but generally do not express desmin (a muscle-specific type III intermediate filament). Two types of myofibroblasts are present in the human intestine: the sub-epithelial myofibroblasts (SEMFs), located primarily in the lamina propria, and the interstitial cells of Cajal (ICC), pacemaker cells responsible for smooth muscle contraction and present in submucosal and muscularis propria layers of the bowel wall.[64] [63,65,66] SEMFs form a three-dimensional network connected to each other by both gap and adherent junctions, and closely interact with the epithelial cell layer through fenestrations in the basement membrane.

Numerous mediators (including cytokines such as TGFβ, connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), IL6 and IL13, discussed in more detail in section 2.1.2.) can promote myofibroblast ECM production and stimulate their proliferation. Additionally, cellular products from injured cells (so-called damage-associated molecular patterns (DAMPs) and microbial products have been shown to activate myofibroblasts.[63,64,67] Myofibroblast activation leads to an increased production of various ECM remodelling enzymes and ECM components such as collagen, fibronectin, glycosaminoglycans and tenascin.[64]

In physiological circumstances, myofibroblast activation is halted when wound healing is completed through necrosis/apoptosis. In fibrosis, however, a derangement of the feedback mechanisms leads

to a prolonged activation and excessive ECM deposition. Although the underlying processes are not completely understood, repeated and chronic injury leading to an unremitting activation of the effector cells is believed to play a key role.[57] Interestingly, in experimental models of liver fibrosis, removal of the fibrosis-inducing agent has been shown to lead to both a reduction in the number of myofibroblasts (by increased apoptosis) and a reversal to a quiescent state (senescence) accompanied by a reversal of fibrotic scar tissue, illustrating the crucial role of myofibroblasts in fibrogenesis.[68] Lastly, inflammation in itself might be important in controlling the fibrogenetic response. In muscle fibrosis for example, TNFα has been shown to induce apoptosis of myofibroblast progenitor cells early in the inflammatory process, limiting fibrogenesis from the start.[69]

Different from other organ systems, intestinal myofibroblasts are derived from a whole range of precursor cells, including mesenchymal cells like fibroblasts and smooth muscle cells (SMC) but also epithelial and endothelial cells (by epithelial/endothelial-mesenchymal transition (EMT/EndoMT)), pericytes, fibrocytes and stellate cells.[63,64]

2.1.2. Fibroblasts

Intestinal fibroblasts are vimentin$^+$, αSMA$^-$, desmin$^-$ mesenchymal cells residing within the connective tissue of the gut and represent a heterogeneous cell population.[63,64] In normal, quiescent circumstances the resident fibroblast expresses only limited actin-based cell-cell and cell-matrix contacts and produces little ECM. Upon tissue injury, fibroblasts become activated and migrate towards the damaged site and starts producing ECM components. Activation is associated with the assembly of cytosolic actin- "stress fibers" which allow for cell motility and connection to the matrix. This fibroblast phenotype (called the protomyofibroblast), although stable in cell cultures, evolves in *in vivo* circumstances to a differentiated myofibroblast by de novo production of αSMA containing stress fibers and is one of the most important sources of myofibroblasts.[70] This transition is controlled primarily by TGFβ, IL1β and mechanical tension (increased matrix stiffness, see further).[63]

Apart from transitioning to myofibroblasts, fibroblasts start to proliferate massively upon tissue injury, controlled by growth factors such as CTGF, PDGF, TGFβ and pro-inflammatory cytokines as IL6 and TNFα.[64] The number of activated fibroblasts present in the damaged tissue site will increase additionally by migration from non-affected sites which is induced by both autocrine stimuli (e.g. fibronectin) as paracrine signals (e.g. TGFβ, PDGF, epithelial growth factor (EGF)), while pro-inflammatory cytokines as TNFα and IFNγ generally inhibit fibroblast migration.[71]

2.1.3. Smooth muscle cells

Intestinal smooth muscle cells (SMCs; vimentin$^-$, αSMA$^+$, desmin$^+$) reside within the muscularis mucosae and are the third phenotype in which intestinal mesenchymal cells can differentiate (aside from (myo)fibroblasts).[64] Under normal circumstances, SMCs produce only limited amounts of ECM proteins but can become activated and even transdifferentiate into myofibroblasts upon tissue injury.[63,66] In an inflammatory environment (e.g. TGFβ and IL1β), SMCs will secrete large amounts of IL6, matrix metalloproteinases (MMPs) and collagens type I, III and IV contributing to fibrogenesis.[64] In both UC and CD, proliferation of SMCs is responsible for the thickening of the muscular layers of the bowel wall and contributes to stricture formation.[63,72]

2.1.4. Stellate cells

Stellate cells (SCs) are mesenchymal precursor cells expressing characterized by Vitamin A expression, αSMA and glial fibrillary acidic protein and are one of the main contributors in liver fibrosis.[63,64] Hepatic stellate cells have been shown to transdifferentiate into myofibroblast-like cells with a strong fibrogenic ability when introduced to an inflammatory environment. Interestingly, they have been isolated in other organs such as the lung, pancreas and gut.[64] In IBD specifically, stellate cells isolated from the mucosa of IBD patients have been shown to have a higher proliferation rate, transdifferentiate into myofibroblasts faster and produce higher amounts of collagen compared to SCs isolated from normal mucosa.[73]

2.1.5. Pericytes

Pericytes are mesenchymal cells surrounding the endothelial cells of capillaries and small blood vessels and play an important role in angiogenesis and endothelial cell differentiation.[64] Expressing αSMA, desmin and endothelin-1 they represent an intermediate phenotype between fibroblasts and vascular smooth muscle cells.[63] During initial phases of fibrogenesis, pericytes are responsible for increased ECM production in the proximity of blood vessels. Additionally, because of their ability to transdifferentiate into myofibroblasts, pericytes represent an important effector cell reserve during fibrogenesis.[74-76]

2.1.6. Fibrocytes

Fibrocytes are circulating, bone marrow-derived mesenchymal progenitor cells bearing features of leukocytes and fibroblasts and expressing markers of both (including cluster of differentiation (CD45), CD34, vimentin and collagen).[77] They constitute 0.5 – 1% of circulating leukocytes, increasing to 6-15%

in inflammatory or fibrogenic circumstances. Fibrocytes migrate to inflamed tissues in a CCR2-mediated way and differentiate into (myo)fibroblasts when exposed to pro-fibrotic cytokines (e.g. TGFβ, IL4, IL13).[63] In a murine CD model, fibrocytes were shown to already appear in the colonic submucosa one week after colitis induction and this preceded the accumulation of αSMA$^+$ myofibroblasts, suggesting fibrocytes as a possible source of myofibroblasts in murine colitis.[78] Little is known, however, about the possible role of fibrocytes in human IBD.[64]

2.1.7. Epithelial and endothelial cells

Epithelial cells are important in the initiation phase of fibrosis and epithelial injury will release pro-fibrotic factors such as TGFβ orchestrating (myo)fibroblast recruitment and activation.[57] Most of the evidence for the role of epithelial cells in initiating fibrosis comes from renal fibrosis: in ischemic, toxic and obstructive models of kidney fibrosis, an association between epithelial cell cycle arrest, production of pro-fibrotic mediators and fibrosis was shown and bypassing this phenomenon by means of a p53 inhibitor reduced interstitial fibrosis. Moreover, selective TGFβ overexpression in tubular epithelial cells is sufficient to induce kidney fibrosis.[79] Also in IPF, fibroblasts foci forming around injured alveolar epithelial cells drive the progression of pulmonary fibrosis.[80] In IBD, inflammatory cytokines such as IL1β, TNFα and IFNγ induce epithelial secretion of TGFβ and TIMP-1 which in turn are able to activate (myo)fibroblasts and stimulate collagen production in an *in vitro* co-culture model.[81]

Epithelial-to-mesenchymal transition (EMT) also contributes to intestinal fibrosis. EMT is a key process in embryonic tissue development in which epithelial cells undergo dramatic changes in cell morphology and function and adopt a mesenchymal phenotype. This transformation process, controlled mainly by TGFβ, is characterized by a disruption of the local basement membrane, subsequent loss of epithelial cell adhesion (apparent by a loss of E-cadherin expression, one of the hallmarks of EMT), *de novo* synthesis of αSMA and rearrangement of cytoskeletal proteins. Eventually the transformed epithelial cells that now have adopted a spindle cell morphology will transmigrate through the basement membrane into the interstitial space and contribute to fibrogenesis.[52,63,64,75,82] In fibrostenotic CD, Scharl et al were able to show presence of EMT hallmarks in fibrotic areas of colon resection specimens suggesting a role of EMT in CD-associated intestinal fibrosis.[83]

Endothelial cells can affect intestinal fibrogenesis in multiple ways. In response to injury, the vascular endothelium will promote vasoconstriction, leading to tissue hypoxia which in turn will activate hypoxia-induced signalling pathways including the hypoxia-inducible factors (HIF-1 and HIF-2).[84]

Hypoxia signalling in turn induces the transcriptional activation a variety of genes, including *TGFB* and collagens from intestinal (myo)fibroblasts.[85,86] Additionally, lactic acid, a by-product of HIF-1 mediated glycolytic metabolism, can stimulate TGFβ secretion.[87] Injured endothelial cells will induce a coagulation response, traditionally one of the first steps in during the wound healing process, that is associated with release of chemotactic factors from platelet granules recruiting innate and adaptive immune cells.[88] Also, injured endothelial cells will secrete inflammatory cytokines including IL6 and IL1β which are prototypical profibrotic cytokines.[89] Lastly, upon stimulation with profibrotic factors, endothelial cells can transform into spindle-shaped, collagen producing mesenchymal cells expressing typical fibroblasts markers (e.g. αSMA, vimentin and fibroblast-specific protein-1 (FSP-1)) while losing expression of typical endothelial markers (CD31 and vascular endothelial cadherin), a process that is called endothelial-to-mesenchymal transition (EndoMT), EndoMT was shown to be present in both the inflammatory mucosa of CD patients and in murine models of intestinal inflammation.[90]

2.1.8. Role of the innate immune system

The innate immune system plays an important role in the initiation of the fibrogenic response. Epithelial injury and invading pathogens result in the release of damage- and pathogen-associated molecular patterns (respectively DAMPs and PAMPs) which can activate innate immune cells through Toll-like receptor (TLR), Nod-like receptor (NLR) and c-type lectin receptors.[91] Chemokine gradients produced by these activated immune cells, damaged epithelial/endothelial cells and local platelet degranulation will further recruit additional innate immune cells which will produce multiple pro-inflammatory as well as pro-fibrotic cytokines such as TGFβ, PDGF and IL6.[63,64]

Macrophages are among the first cell types to be recruited to the injured tissue site. To date, a large variety of functional macrophage subtypes has been described, and their relative concentrations play an important role in determining whether normal tissue repair or fibrosis follows the injury. M1 pro-inflammatory macrophages are activated by IFNγ, TNFα or bacterial products and secrete IL1, IL12, IL23, TNFα and reactive oxygen species (ROS).[91] They promote fibrosis by activating myofibroblasts, ensuring additional tissue damage, and by interfering with myofibroblast apoptosis.[63,92] Enhanced phagocytosis and exposure to IL4/IL13 when inflammation starts to resolve or becomes chronic, will induce a M2a macrophage phenotype that produces crucial pro-fibrotic mediators as TGFβ, CTGF and PDGF. M2c or so-called regulatory macrophages appear following IL10 exposure and are considered anti-inflammatory and anti-fibrotic: they produce IL10 and Arginase-1 which will inactivate both myofibroblasts and M1/M2a macrophages.[63,92]

The role of other leukocytes in fibrosis is less established and more circumstantial: neutrophils are recruited early in the inflammatory response and are essential for debris removal and bacterial killing. Persistent activation promotes further tissue damage and release of pro-fibrotic cytokines activating myofibroblasts. Eosinophils and mast cells on the other hand produce TGFβ and IL13 and recruit inflammatory leukocytes, further promoting fibrogenesis. The exact role of basophils in intestinal fibrosis remains unclear, but may act as a source of type 2 cytokines (IL4, IL13).[63]

2.1.9. The adaptive immune system and intestinal fibrosis

IBD is characterized by aberrant adaptive immune responses that have a major influence on fibrogenesis.[91] The T helper 1 (Th1) response, with IFNγ production as its hallmark, is generally considered anti-fibrotic. IFNγ interferes with TGFβ signalling and reduces TGFβ and CTGF expression.[93] In murine models of liver fibrosis, IFNγ knock-out mice develop a more severe phenotype which can be reversed by external IFNγ administration. In a renal fibrosis model, selective IFNγ administration to renal myofibroblasts ameliorated fibrosis and reduced collagen production.[94]

The Th17 response, however, has been shown to enhance fibrogenesis as it promotes myofibroblast TGFβ, CTGF and collagen production. Interestingly, stenotic CD tissue samples show high levels of IL17A, one of the key cytokines produced by Th17 cells. Additionally, myofibroblasts isolated from these strictures showed an enhanced production of collagen and MMPs upon IL17A stimulation.[95] IL22, however, also produced by Th17 cells, plays an important anti-fibrotic role in lung fibrosis.[96 97]

Although abnormal Th2 responses (mainly characterized by IL4 and IL13 production) are typical hallmarks of UC, they also play an important role especially in long standing CD.[98] Both cytokines share a common IL4Rα receptor signalling pathway through STAT6, but evidence from experimental colitis in double IL4/IL13 knock-out mice showed that IL13 elicits the more pronounced fibrogenic response.[99] Both cytokines can directly activate myofibroblasts, resulting in an enhanced collagen and fibronectin production.[100] In addition, IL13 induces TGFβ production from intestinal macrophages orchestrating further pro-fibrotic signaling.[91]

2.2. Major molecular mediators of fibrosis

Fibrogenesis in the intestine is orchestrated by paracrine factors secreted by both immune and non-immune cells and autocrine signals derived from myofibroblasts themselves. Together they create a pro-fibrogenic milieu which will activate ECM productions from myofibroblasts.

2.2.1. Growth factors

2.2.1.1. Transforming growth factor β (TGFβ)

The prototypical pro-fibrotic protein TGFβ is primarily produced by macrophages and (myo)fibroblasts. It is secreted in an inactive form, bound to the latency-associated protein (LAP).[63] To exert its biological function, TGFβ needs to dissociate from LAP, a process mediated by various proteases such as plasmin, thrombin, MMPs.[101] Canonical intracellular SMAD signalling is activated by binding of TGFβ to one of its three receptors (TGFβRI, II or III) and results in phosphorylation of SMAD2/3 which in turn induces phosphorylation of SMAD4. The SMAD2/3-SMAD4 complex translocates to the nucleus and regulates transcription of TGFβ target genes including several collagen genes (e.g. *COL1A1, COL3A1*), αSMA, *CTGF*, *TIMP* and over 60 other ECM-related genes.[102] SMAD-dependent TGFβ signalling also is involved in myofibroblast activation, induces EMT/EndoMT and inhibits MMP production. SMAD6 and 7 are part of a negative feedback mechanism in canonical TGFβ signalling and antagonize phosphorylation of SMAD2/3 by the receptor complex.[56,63] Non-canonical, SMAD-independent TGFβ signalling includes the ERK/c-jun/p38 MAP kinase and Rho/ROCK pathway both of which are also involved in fibrogenesis (see section 3) (**Figure 2**). Three mammalian isoforms of TGFβ exists (TGFβ 1, 2 and 3) with TGFβ1 and 2 promoting fibrogenesis while TGFβ3 has important anti-fibrotic effects. [103,104]

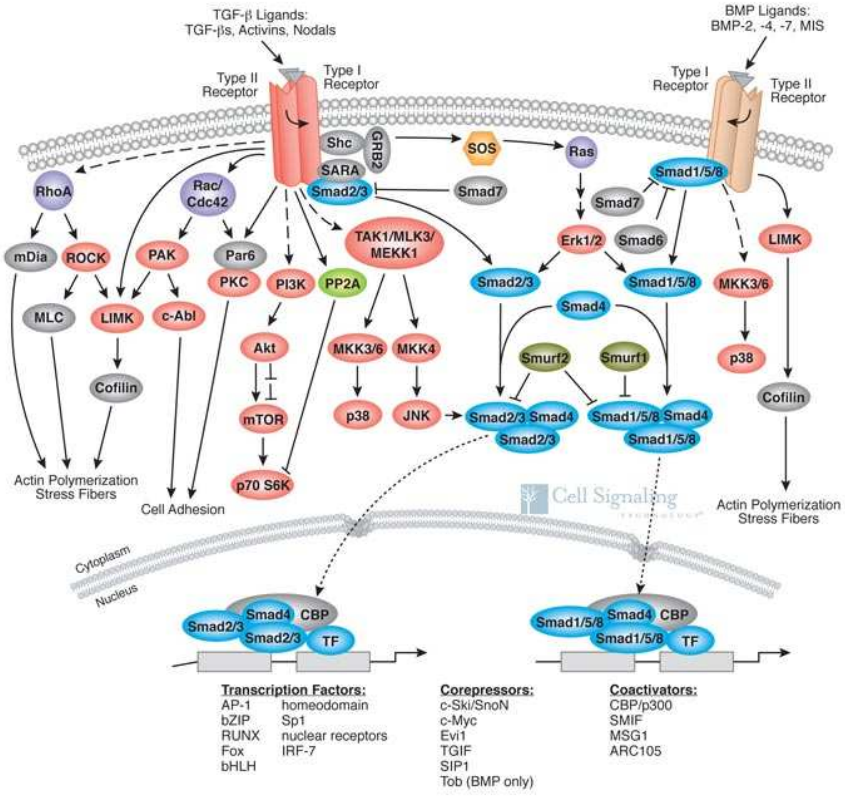

Figure 2 – TGFβ signalling pathway. Overview of the most important downstream mediators of TGFβ. [From: Human Pathology 2017]

TGFβ/SMAD signalling plays a crucial role in intestinal fibrosis and fibrosis in other organ systems.[50,56,63,75,101] Both TGFβ and its receptors have been shown to be overexpressed in fibrostenotic CD.[105] Adenovirus-mediated overexpression of TGFβ in the murine colon leads to fibrosis, while SMAD3 knock-out mice show resistance to TNBS-induced fibrosis.[106,107] Experimental SMAD3 overexpression or loss of SMAD7 signalling, both interfering with the normal TGFβ/SMAD pathway, ameliorates fibrosis in several organ systems.[108-111] Moreover, fibrostenotic CD tissue exhibits decreased SMAD7 and increased SMAD2/3 signalling, underscoring the importance of canonical TGFβ signalling in intestinal fibrosis.[105]

2.2.1.2. Activins

Three isoforms of activin exist (Activin A, B and AB) and as members of the TGFβ superfamily can activate both the canonical SMAD and MAP kinase pathway. They play an important role in tissue repair and fibrosis, and increased levels in bowel resection specimens of IBD patients have been found.[112]

2.2.1.3. Connective tissue growth factor (CTGF)

CTGF is a downstream mediator of SMAD-dependent TGFβ signalling and is often co-expressed with TGFβ in fibrotic disorders.[64] A cysteine-rich peptide, CTGF is secreted mainly by (myo)fibroblasts and is pivotal for ECM production from connective tissue cells.[63] Interestingly, it does not appear to act on epithelial or immune cells. In fibrostenotic CD, CTGF was found to be upregulated in stenotic resection specimens.[113]

2.2.1.4. Platelet-derived growth factor (PDGF)

Megakaryocytes are the main source of PDGF, but also (myo)fibroblasts, smooth muscle cells and macrophages produces PDGF in response to TGFβ, IL6 or TNFα stimulation.[64] Its expression is increased in the inflamed mucosa of IBD patients and PDGF has been shown to activate, enhance proliferation and migration of (myo)fibroblasts and intestinal smooth muscle cells.[114]

2.2.1.5. Other growth factors involved in intestinal fibrosis

Insulin-like growth factor (IGF) is upregulated in CD and models of intestinal fibrosis.[56,115,116] IGF is secreted by mesenchymal cells upon stimulation with pro-inflammatory cytokines and induces proliferation of both (myo)fibroblasts and epithelial cells and increases collagen production.[117]

Fibroblast growth factor (FGF) belongs to a family of heparin-binding proteins that have diverse functions in fibrogenesis.[63] Basic FGF (bFGF) induces (myo)fibroblasts and smooth muscle cell proliferation, stimulates collagen production from these cells and works synergistically with CTGF.[101] Moreover, bFGF serum levels are elevated in fibrostenotic CD and correlate with the thickness of the bowel wall, suggesting an essential role in intestinal fibrosis.[118] FGF2 has been associated with cardiac fibrosis, while FGF23 seems to be involved in both cardiac and renal fibrosis. [119] Conversely, FGF16 and 21 have been shown to antagonize fibrogenesis by competing with FGF2-receptor binding.[120]

Lastly, the **epidermal growth factor** (EGF) has an established role in IPF, stimulating collagen production, proliferation and migration in pulmonary fibroblasts.[121] Conversely, although EGF has been isolated from the intestine, its role in intestinal fibrosis is unclear. In TNBS colitis, EGFR has been shown to be upregulated, but external administration of EGF ameliorates colitis.[122] In human fibroblasts, however, EGF has been shown to stimulate both (myo)fibroblast proliferation and migration.[123]

2.2.2. Profibrotic cytokines

2.2.2.1. Profibrotic interleukins

IL1 belongs to a family of rapid-response, pro-inflammatory cytokines that are secreted in the early phase of inflammation and tissue damaging.[64] In chronic inflammatory disorders, IL1 contributes to fibrogenesis by activating (myo)fibroblasts, inducing MMP secretion and interfering with ECM turnover.[124] Additionally, IL1 plays, together with TGFβ and IFNγ, an important role in the regulation of EMT.[125] **IL5** on the other hand is an example of a fibrosis-amplifying cytokine: it facilitates secretion of TGFβ and IL13 but in itself does not have a fibrogenetic role.[65]

Aside from a pivotal involvement in inflammation **IL6** also has strong pro-fibrotic properties.[126] Levels are markedly increased in CD and IL6 has been shown to activate mesenchymal cells, increase TGFβ expression and stimulate (myo)fibroblast proliferation.[127] Additionally, in an allograft-model neutralization of IL6 prevented cardiac fibrosis.[128] **IL7**, however, has an anti-fibrotic role by

upregulating SMAD7 expression and interfering with TGFβ-related (myo)fibroblast activation and collagen deposition.[129] **IL10** is the archetypical anti-inflammatory cytokine and also slows fibrosis progression.[65]

IL4 and **IL13** are secreted by Th2 cells and have prominent roles in fibrogenesis which have been discussed earlier (see 2.1.9). **IL21/22** are strongly linked with CD-associated fibrosis. IL21 is produced in excess in CD and has been shown to enhance pro-fibrotic Th2 signaling.[130] Additionally, IL21 augments IL4 and 13 receptor expression in macrophages and stimulates MMP secretion from (myo)fibroblasts).[131] In contrast, IL22 inhibition stimulates collagen deposition in pulmonary fibrosis model, suggesting a protective role.[96]

IL33 is a member of the IL1-family of pro-inflammatory cytokines and was shown to be upregulated in UC erosion-associated fibroblasts, but not in CD. IL33 is induced upon TLR3 stimulation and induces (myo)fibroblasts activation, suggesting a possible role in UC related fibrosis.[132] Lastly, the **IL23/IL17** axis might play a role in intestinal fibrogenesis, which has been covered in chapter 2.1.9.

2.2.2.2. Tumor necrosis factor α

TNFα, produced primarily by macrophages and T cells, is one of the best-known cytokines involved in IBD. Its role in intestinal fibrosis, however, is dual. It is generally considered to stimulate fibrogenesis by enhancing (myo)fibroblast proliferation and collagen secretion through TNF receptor 2 (TNFR2) signalling. Moreover, TNFα induces TIMP-1 expression, reduces MMP-2 activity resulting in collagen deposition and it stimulates production of other pro-fibrotic cytokines such as IL6 and IL13.

Conversely, studies in muscle fibrosis have shown that TNFα induces (myo)fibroblast apoptosis and is crucial for terminating the wound healing process with TNFα neutralization enhancing fibrosis in this particular fibrosis model.[69] This dual role of TNFα probably explains the inability of TNFα antagonists to reduce intestinal fibrosis in IBD despite their strong anti-inflammatory actions.

2.2.2.3. Interferon

IFNγ supresses formation of fibrotic tissue by direct interference with SMAD3 phosphorylation and by induction of SMAD7 expression thereby antagonizing TGFβ signalling. Additionally, IFNγ inhibits fibroblast proliferation and reduces collagen production from (myo)fibroblasts.[133] Lastly,

IFNγ prevents fibroblast migration by interfering with cytoskeleton assembly.[134] Despite its numerous anti-fibrotic properties, clinical trials using IFNγ have delivered disappointing results.[135]

2.2.2.4. Chemokines

Chemokines are leukocyte chemoattractants and recruit immune cells to the injured tissue. Certain type of chemokines, however, are necessary for fibrogenesis and disruption of these pathways reduces fibrosis. Specifically, CC and CXC chemokines such as CCL2 (or monocyte-chemoattractant protein 1 (MCP1), CCL3 (macrophage inflammatory protein (MIP1)), CCL4 (MIP1b) and CCL20 (MIP3a) are elevated in CD related fibrosis.[63-65,136]

2.2.3. Important signaling receptors and pathways involved in intestinal fibrosis

Aside from cytokines and growth factors other signalling pathways and their activators can stimulate (myo)fibroblasts and enhance fibrogenesis. A short overview of the best-known molecular factors is given below.

2.2.3.1. Renin-angiotensin system (RAS)

The RAS system is a hormone system essential to human blood pressure regulation. Its components play an important role in cell growth, inflammation, ECM production and fibrosis and all of its components are found locally within the gut. Angiotensin II is the main effector of the RAS system and stimulates fibrogenesis mainly by increasing TGFβ production. Both TGFβ and Angiotensin II are overexpressed in fibrostenotic CD [137,138] and daily administration of captopril (an angiotensin converting enzyme inhibitor) in a chronic TNBS colitis model decreased colonic deposition of collagen and reduced TGFβ expression.[139]

2.2.3.2. Peroxisome proliferator activator receptors (PPAR)

PPARs are nuclear receptors involved in cell growth, differentiation and metabolism. PPARγ is expressed in intestinal macrophages, dendritic cells, B and T cells.[56] PPARγ activation directly antagonises SMAD3 and downregulates expression of CTGF thereby counteracting TGFβ-induced fibrogenesis.[140] Reduced PPARs activation results in collagen deposition, while overexpression reduces fibrosis.[141,142]

2.2.3.3. PAMPs and TLRs

Luminal bacteria express PAMPs including lipopolysaccharide (LPS), bacterial DNA and double stranded RNA that can bind and activate pattern recognition receptors such as TLRs. TLRs are expressed on both immune and non-immune cells and immune cell TLR activation (via MyD88) leads to an inflammatory response pivotal in the pathogenesis of IBD.[63] Fibroblasts express TLR and stimulation with LPS (via TLR4), the gram-positive lipoteichoic acid (TLR2 activation) and flagelin (TLR5) can promote myofibroblast formation and ECM production.[143] [144]

2.2.3.4. DAMPs

Damage associated molecular patterns (including DNA, RNA, ECM fragments and metallothioneins) are released upon cell injury and cause a sterile inflammation. The exact role of DAMPs in intestinal fibrosis and whether they contribute to fibrogenesis is unknown. However, inhibiting the release of high-mobility group box 1 (HMGB1), a DNA binding protein, has been shown to prevent development of renal fibrosis.[56,145]

2.2.3.5. Endoplasmic reticulum stress

Endoplasmic reticulum stress or ER stress is a cellular stress mechanism resulting from the misfolding of proteins and it drives the cell towards apoptosis.[146] Not much is known about the role of ER stress in intestinal fibrosis, however, ER stress has been shown to activate EMT, TGFβ/SMAD and the Wnt/β-catenin pathway, all key elements in fibrogenesis.[147,148] Additionally, by inducing apoptosis, ER stress facilitates ECM remodelling.[56]

2.2.3.6. Signaling pathways involved in embryonic development

Overactivation of several pathways involved in embryonic development and progenitor cell differentiation appear to play a major role in fibrogenesis. For instance, activation of the **hedgehog signalling pathway** has been shown to lead to myofibroblast activation, ECM deposition and is involved in EMT.[149] Conversely, inhibition of this pathway is anti-fibrotic in murine models of systemic sclerosis.[150] The **Wnt/β–catenin pathway** is involved in EMT, is activated by TGFβ signalling in a p38-dependent manner (via lowered expression of the Wnt-antagonist DKK-1) and activation results in an increased ECM deposition.[56,151] Lastly, the embryonic important **Notch signalling** pathway is overactivated in fibrosis and Notch signalling inhibition ameliorates hepatic fibrosis, reduces EMT and lowers TGFβ production.[152]

2.2.3.7. Molecular pathways of aging in intestinal fibrosis

Fibrosis is typically a complication of longstanding disease and aging in itself can promote fibrogenesis. Accumulation of advanced glycation end-products (AGEs), formed by the non-covalent binding of reducing sugars to amino acid groups in proteins, promotes EMT, ECM accumulation and increases accumulation of active myofibroblasts via the receptor of advanced glycation end-products (RAGE).[153] Moreover, RAGE expression levels have been shown to be increased in active IBD.[154]

Telomere shortening aggravates with ageing and circulating leukocytes with shortened telomeres are found in high quantity in patients with IPF.[155] These findings could be attributed to mutations in the gene encoding for the telomerase reverse transcriptase (TERT), an enzyme crucial in the maintenance of telomere length and a reduced TERT activity has been shown to active (myo)fibroblasts.[156] Interestingly, TGFβ and ROS have been shown to participate in telomere shortening, suggesting an ongoing cycle of telomere shortening, myofibroblast activation and production of pro-fibrotic factors.[136]

2.3. Inflammation independent mechanisms of fibrosis

Previously discussed mechanisms of fibrogenesis are highly associated with inflammation. Curiously, however, current anti-inflammatory therapies do not seem to adequately prevent intestinal fibrosis and fibrosis progresses even when inflammation is suppressed, suggesting inflammation-independent autopropagation of fibrogenesis.[50] The extracellular matrix and its rigidity play an important role in this process (**Figure 3**).

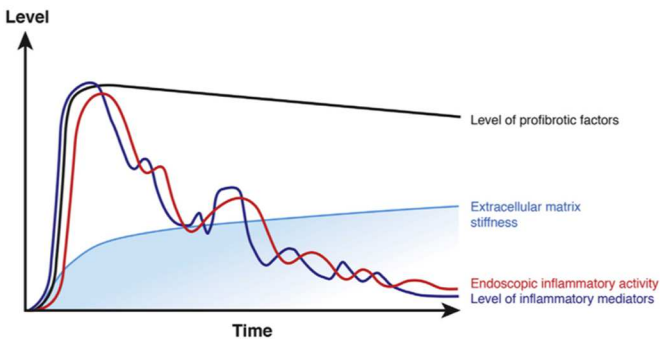

Figure 3 - Intestinal fibrosis becomes autopropagatory over time. Illustration of evolution of fibrosis over time. Even when inflammation is suppressed, pro-fibrotic mediators remain elevated and this is importantly linked to matrix stiffness. [From Rieder et al.[50]]

2.3.1. The extracellular matrix

Within the bowel wall, the lamina propria and submucosa consist of ECM tissue, a large non-cellular environment which gives the bowel tissue elasticity, compression and tensile strength. The ECM consists for more than 90% of water, giving the tissue its viscosity with the remaining being made up

out of macromolecules such as collagen, proteoglycans and glycoproteins all produced by fibroblasts and other mesenchymal cells.[157]

Fibrillary collagens give the ECM tensile strength and consist of repetitive amino acid motifs containing glycine-proline-X or glycine-X-hydroxyproline forming α-chains organized in triple helix configurations. Over 40 α chain encoding genes exist which can assemble into 28 known types of collagen. In the gut, mainly collagen I (mucosa), III (submucosa) and IV (basement membrane) are found. In fibrotic CD, expression of these collagens is highly increased.[157,158]

Glucosaminoglycans (GAGs) (such as heparin and heparan sulphate) are large unbranched chains of polysaccharides containing a net negative charge resulting in an extended structural conformation that attracts and holds water. Bound to protein cores, they form proteoglycans which fill in the ECM space between collagen fibers. Apart from providing hydration to the tissue, GAGs also bind and release growth factors such as TGFβ. Hyaluronic acid is an atypical GAG which is not bound to a protein core but can adopt extremely large molecular weights and is a major source of tissue hydration.[157,158]

Glycoproteins are another form of ECM proteins involved in cell-matrix interactions. Laminins are the most abundant form of glycoproteins, are mainly found in the basement membrane of the ECM and interact with the epithelium by the integrin α6β4 receptor. Fibronectin is a fibril forming glycoprotein connecting cells to other ECM proteins. During fibrosis, fibroblasts will produce a different form of fibronectin (fibronectin ED-A) which will stimulate myofibroblast differentiation.[157,158]

2.3.2. Increased matrix stiffness as a source of fibrosis autopropagation

After tissue injury, the wound healing process, for reasons which are not completely understood, leaves a newly produced ECM that is less organized and more rigid. When fibrosis develops, this matrix stiffness is even further increased by an increased abundance of ECM fibrils and increased ECM cross-linking induced by TGFβ[159] In fibrostenotic CD, for instance, the bowel wall is ten times less compliant compared to normal healthy tissue.[160]

The increased matrix stiffness can both induce and maintain fibrogenesis through direct activation of (myo)fibroblasts by mechanotransduction but also by releasing growth factors and DAMPs.

2.3.2.1. Mechanotransduction and myofibroblast activation

Fibroblasts reside within the ECM, closely attached to its components and use this connection for mechanical sensing of the environment. Focal adhesion complexes located in the cell membrane are sites where ECM proteins connect to the fibroblast cytoskelet through integrins, transmembrane heterodimers composed of an α and β subunit. Over 24 different mammalian integrins exist, however α5β1 and αv are the most important ones involved in mechanotransduction and are expressed abundantly in intestinal fibroblasts.[161,162] At the focal adhesion complex, the β subunit of the integrins connects to F-actin stress fibers of the fibroblast cytoskeleton through several adaptor proteins (e.g. vinculin, talin, paxilin). (**Figure 4**)

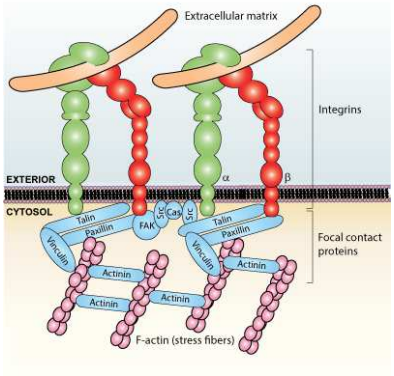

Figure 4 – Schematic representation of a focal adhesion complex. FAK = focal adhesion kinase, SRC = SRC kinase family. [From Laukens et al.]

In the quiescent state, fibroblasts do not express these stress fibers resulting in only loose cell-matrix connections. Upon tissue injury, fibroblasts become activated and F-actin stress fibers (a hallmark of the protomyofibroblast) will be formed that allow for stronger cell-matrix connections.[70,163] As the fibroblast migrates over the ECM, these connections will create tension (so-called pre-stress), that will be compensated by formation of additional stress fibers and adhesion complexes. As the matrix becomes less resilient with increasing fibrosis, more pre-stress is generated and the fibroblast cytoskeleton will adjust accordingly by increasing the number of F-actin stress fibers.[163] Biochemically, the increasing number of F-actin stress fibers is the result of concomitant activation of focal adhesion kinases located within the adhesion complex that in turn will activate the Rho/ROCK signalling pathway resulting in an increased polymerisation of G-actin to F-actin (see chapter 3).[164]

Simultaneously, the transition from globular (G-)actin to filamentous (F-)actin stress fibers will activate pro-fibrotic signalling pathways. G-actin monomers sequester the myocardin-related transcription factor (MRTFA and B) to the cytosol. Upon polymerisation of monomeric G-actin into F-actin, MRTF is released and translocates to the nucleus were it will act as a cofactor for the serum response factor (SRF) and activate transcription of pro-fibrotic genes such as TGFβ, fibronectin, collagen and ACTA2 (encoding αSMA).[165] The latter will eventually result in differentiation from protomyofibroblast to

myofibroblast that has more pronounced contractile capabilities, essential in late phase wound healing.[166]

2.3.2.2. Matrix stiffness and increased release of growth factors

TGFβ is secreted into the matrix in inactive form, sequestered within the large latency complex consisting of the latency-associated propeptide (LAP) and the TGFβ binding protein-1 (LBTP1). LAP is capable of binding integrin (mainly αv integrin) resulting in connection between the ECM and the latency complex. In a compliant matrix, the latency complex will be dragged along unharmed upon fibroblast migration. With increased matrix stiffness, however, the integrin-mediated force can induce a conformational change in LAP, liberating TGFβ to exert its biological function.[167]

Finally, TGFβ in the ECM is bound to several proteoglycans such as decorin and fibronectin affecting its bio-availability.[168]

2.3.2.3. Release of DAMPs by the ECM

Both damage to the ECM and fibroblast-induced remodelling can release DAMPs from the ECM which will further activate fibroblasts. For example, hyaluronidases secreted by several cells during inflammation including fibroblasts, will digest hyaluronic acid into shorter segments that will in turn activate fibroblasts through TLR2 and TLR4 signaling.[169] Similarly, degraded fibronectin can also stimulate fibroblasts through TLR4 activation.[170]

2.3.2.4. MMP/TIMPs and ECM remodelling

The ECM is a dynamic structure that is constantly being remodelled. Matrix metalloproteinases (MMPs) are zinc and calcium dependent ECM degrading endopeptidases that can collectively degrade all ECM products. Additionally, they have been shown to activate (or in some instances neutralise) growth factors and cytokines. Numerous types of MMPs have been described including collagenases (MMP-1,-8,-13,-18), gelatinases (MMP-2,-9), stromelysins (MMP-3,-10,-11), matrilysin (MMP-7), elastase (MMP-12) and membrane-bound MMPs (MMP-14,-15,-16,-17,-24,-25). Their function is antagonized by tissue inhibitors of MMPs (TIMPs 1-4). Myofibroblasts, epithelial cells, leukocytes and macrophages are the most important sources of MMPs and TIMPs.[171,172]

Although the balance of MMP/TIMP seems to be disturbed in IBD, their exact role in the pathophysiology of intestinal fibrosis is incompletely understood.[56] Generally, MMPs appear to be

upregulated in both murine colitis models and human IBD. In the mucosa overlying CD strictures, expression of TIMP-1, MMP-3 and -12 is increased in comparison to mucosa overlying non-fibrotic sections of the bowel.[105] In murine models of intestinal fibrosis, MMP-1 but not MMP-3 levels are elevated.[173] In IBD in general, MMP-3 and -9 are associated with mucosal damage and fistulas, MMP-1,-3 and -13 with intestinal ulcers and MMP-10, -11 with epithelial dysfunction. [56,171,172] TGFβ increases TIMP-1 production, while downregulating expression of MMP-1.[56] However, MMP/TIMP metabolism is complex, incompletely understood and stating fibrosis results from a reduced MMP (or enhanced TIMP) activity is incorrect. This was demonstrated nicely in a murine colitis model where MMP-9 inhibition actually prevented fibrosis.[174] TIMP-1 knock-out mice, however, do develop less severe fibrosis when challenged with DSS.[175]

2.4. The role of microbiota

Several lines of evidence suggest a role for the intestinal microbiota in fibrogenesis. First of all, several genetic variants associated with fibrostenotic disease are located within genes encoding for bacterial receptors (e.g. NOD2 and TLR4, see section 3). Secondly, antibodies directed against microbial antigens such as anti-Saccaromyces cervisiae antibodies (ASCA) and anti-glycan antibodies (e.g. CBir1, OmpC, anti-I2) correlated with more complicated CD disease including fibrostenosis.[176] Thirdly, fibroblasts express TLR and NLR and can be activated by bacterial products.[56]

Murine models provide some additional evidence for a role of the microbiota in fibrogenesis. For instance, in a spontaneous ileitis model, SAMP1/YitFc mice do not develop fibrosis in germ-free conditions (see section 2.8).[56] Conversely, in another model in which small bowel was transplanted to the neck of the rats and thus eliminating the microbial component, fibrosis still developed rapidly under stimulation with TGFβ. Interestingly, in the same model there was no difference in development of fibrosis between Myd88-knock out (interfering with TLR signalling) and wild type mice, suggesting that at least in this model fibrosis was independent of the innate immune system.[177]

In liver fibrosis, translocation of bacterial products through the portal vein has been shown to activate hepatic stellate cells and contribute to fibrogenesis.[178] Similarly, in cystic fibrosis, dysbiosis is present and correlates with disease severity, both implicating a role for the microbiota in fibrosis.[179]

However, to date no specific 'fibrostenotic' microbial signature has been identified and the exact contribution of the microbiota to intestinal fibrosis remains to be elucidated.

2.5. Adipose tissue

The mesenteric adipose tissue (MAT) is a loose connective tissue located within the mesentery, a double fold of the peritoneum attaching the intestines to the abdominal wall, and contains adipocytes, pre-adipocytes, fibroblasts, macrophages and leukocytes. It functions as an energy regulator but additionally has important immunological and hormonal properties that seem to play a role in intestinal fibrosis.[180]

Macroscopic changes to the MAT can be seen in over 50% of CD patients with fat tissue extending from the mesentery and engulfing the bowel, a phenomenon called “creeping fat”. It correlates with inflammation severity and is most frequently seen around the terminal ileum. Interestingly, bowel segments with creeping fat very frequently show abnormal collagen depositions and strictures.[181] Additionally, in a paediatric population increased MAT volumes were shown to correlate with a more complicated disease course.[182] Taken together these data suggest a relationship between MAT abnormalities and intestinal fibrosis.

One way how the MAT can affect intestinal fibrosis is through adipocytes and their release of adipokines such as leptin and adiponectin. Leptin is a cytokine-like protein, induces a Th1 immune response and is generally considered pro-fibrotic. Leptin-deficient mice for instance develop less severe liver fibrosis.[56,183] Adiponectin on the other hand has important anti-inflammatory properties and antagonises TNFα. Adiponectin knock-out mice develop excessive liver fibrosis while administration of recombinant adiponecin alleviates these changes, suggesting an anti-fibrotic role.[181,184] However, no direct data about the role of these adipokines in CD-related fibrosis are available. C1q/TNF-related protein-3 (CTRP-3), on the other hand, is an adipokine expressed in CD with important anti-fibrotic properties. CTRP-3 has been shown to inhibit TGFβ, CTGF and collagen release from intestinal fibroblasts. [56,185]

Adipose tissue macrophages (ATM) and T lymphocytes (ATT) can additionally explain the role of MAT in intestinal fibrosis as ATMs and ATTs isolated from CD patients were found to produce more pro-inflammatory and pro-fibrotic cytokines like IL6, IL4 and IL13 compared to cells isolated from non-CD patients. Moreover, in creeping fat specifically, a larger number of pro-fibrotic M2 macrophages is present.[180,186]

Lastly, the emergence of creeping fat has been suggested as an anti-microbial defence strategy, isolating an affected bowel segment and preventing bacterial translocation. The fact that adipocytes

bear functional PPRs and can transform into macrophages with a direct anti-microbial activity supports this theory. Creeping fat-induced fibrosis could then be seen as a bacterial containment strategy.[56,187]

2.6. Autophagy and its role in fibrotic diseases

Autophagy is an evolutionary preserved cellular homeostasis mechanism which under basal conditions traffics damaged organelles targeted for destruction to the lysosomes for degradation. Cellular starvation and inhibition of the mammalian target of rapamycin (mTOR) are the most important inducers of autophagy and liberate energy by autodegradation.[188]

Autophagy requires the coordinated action of autophagy-related proteins (ATG). Cellular starvation or mTOR inhibition leads to activation of the unc 51 like kinase (ULK) 1, Atg13 and FIP200 complex necessary for autophagy initiation. Recognition of cellular components targeted for autophagy is mediated by the cytosolic adaptor protein p62/sequestosome 1 which binds to the lipidate microtubule-associated protein 1 light chain 3 or LC3 and delivers the structure to the pre-autophagosomal complex. Next, autophagy proteins are recruited during the nucleation phase coordinated by the beclin-1 - phosphatidyl-inositol 3 kinase (PI3K) - vacuolar sortin protein 34 (vsp34)-p150 complex resulting in the formation of an isolation membrane. The origin of this isolation membrane is incomplete understood but is believed to originate from the endoplasmic reticulum or the Golgi apparatus. Elongation and complete engulfing of the target requires activation of both the Atg12-Atg5-Atg16l1 complex and concomitant conjugation of LC3-I to phosphatidylethanolamine forming LC3-II. Atg7 is necessary for both processes. The resulting autophagosome (with a characteristic double layer membrane) is then transported along the cytoskeletal network to the lysosomes. Fusion of both structures results in the formation of the autolysosome in which the target is destructed (**Figure 5**).[188-190]

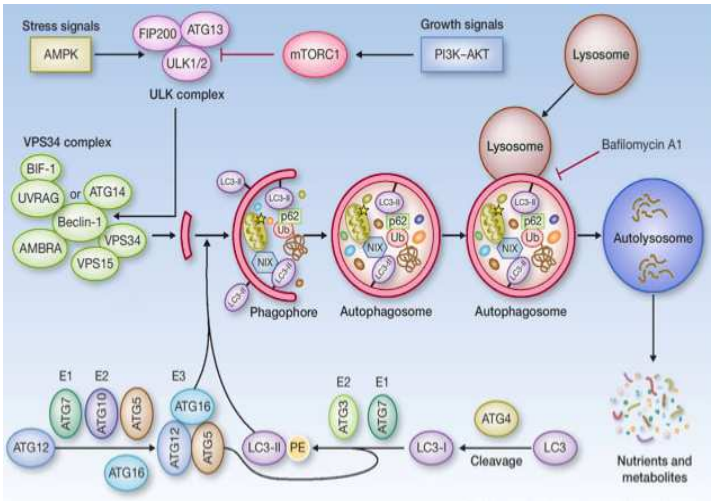

Figure 5 – Illustration of the autophagy pathway. Cellular stress signals or mTOR inhibition recruit the ULK complex essential for autophagy initiation. The ULK complex facilitates assembly of the VPS34 complex which forms an isolation membrane around cellular targets identified by p62-LC3 binding. Elongation and formation of the autophagosome is mediated by the Atg12-Atg5-Atg16l1 complex which then fuses

with the lysosome to form the autolysosome in which the cellular target is destructed. [Adapted From Cicchini et al.[191]]

Autophagy has been implicated in IBD mainly through genetic association studies. *ATG16L1*, involved in autophagosome formation and *IRGM*, necessary for viral and mycobacterium-related autophagy were identified as susceptibility genes for IBD, suggesting a possible role of autophagy in IBD pathology (see section 3). Defective autophagy has been recognised to contribute to IBD by interfering with intracellular bacterial killing, antimicrobial peptide secretion by Paneth cells, goblet cell function, pro-inflammatory cytokine production by macrophages, antigen presentation by dendritic cells and the ER stress response in enterocytes.[188,192]

Few studies have evaluated the relationship between intestinal fibrosis and autophagy. Some evidence, however, is available from other fibrotic disorders, although not always consistent with both up- and down-regulated autophagy being associated with fibrogenesis.

One mechanism by which defective autophagy could play a role in fibrosis is through its role in the elimination of misfolded collagen proteins. Defective autophagy leads to the accumulation of toxic aggregates in the ER promoting fibrogenesis.[189] In cardiac fibrosis, defective autophagy increases heart failure progression and accumulation of interstitial fibrosis and this is accompanied by an accumulation of misfolded aggregates in the cardiomyocytes.[193] In renal murine mesangial cells, beclin-1 deficiency leads to collagen accumulation while induction of autophagy promotes collagen degradation.[194] Similarly, in hypertrophic scar tissue beclin-1 and LC3 levels were lower compared to normal skin.[195]

Upregulated autophagy on the other hand could also play a role in fibrogenesis, by increasing fibroblast survival. In liver fibrosis for instance, hepatic stellate cells continued activation is associated with a dysregulated/increased autophagy providing energy by lipid droplets catabolism.[196] Similarly, in rheumatoid arthritis, survival of synovial fibroblasts is dependent on increased autophagy handling the removal of misfolded proteins.[197]

Several cytokines involved in fibrogenesis can affect the autophagic response. TGFβ for instance has dual effects on autophagy and can both increase (through SMAD-related activation of Atg5 and 7) and decrease the autophagic response (through the PI3K/AKT/mTOR pathway). The type 2 cytokines IL4 and IL13 are known to promote autophagy, while type 1 cytokines as IFNγ and TNFα impair the autophagic response.[189,198]

Taken together, although the exact role of autophagy in fibrotic diseases is not completely understood, an impaired autophagic response seems to promote fibrogenesis. Importantly, in systemic sclerosis patients, treatment with rapamycin (a powerful autophagy inducer) was shown to reduce progression of fibrosis.[199]

2.7. Animal models to study intestinal fibrosis

To study the pathophysiology of intestinal fibrosis several animal models can be used. However, no ideal model that captures the recurring and progressive nature of IBD fibrosis exists to date. An overview is given in Table 1.

2.7.1. Spontaneous fibrosis models

The SAMP1/Yit mouse strain is probably the most representative fibrosis model available. Generated by mating of a senescence-accelerated mouse line, intestinal inflammation that is segmental, transmural and includes granulomas develops by week 10. While Th1 cytokines dominate the induction phase, the chronic phase is characterized by a dominant Th2 response and results in segmental fibrosis and even luminal strictures.[200] The low breeding rate of these mice and the fact that they are not commercially available however limits their applicability.[201]

2.7.2. Chemically induced models

2.7.2.1. Chronic dextran sulphate sodium-induced colitis

Dextran sulphate sodium (DSS) is a sulphate polysaccharide which, when administered in the drinking water, induces a highly reproducible colitis with bloody diarrhoea, ulcerations and weight loss. DSS has a toxic effect on the mucosal barrier, leading to a disruption of the epithelial barrier, translocation of luminal microbiota or antigens, which subsequently activates macrophages that induce intestinal inflammation. Mice lacking T or B cells still develop colitis, suggesting a predominant effect of the innate immune system. Interestingly, although inflammation is importantly influenced by the microbial composition, even germ-free mice develop colitis. [202]

In certain mice strains (e.g. C57BL6), chronic administration of repeated cycles of DSS induces intestinal fibrosis, characterized by an increased deposition of collagen in the mucosa and submucosal layers, thickening of the muscle layers and myofibroblast infiltration.[56,203] Although the mechanism of induction is questionable in its relevance to CD related intestinal fibrosis, chronic inflammation and

resulting fibrosis induced by repeated cycles of DSS strongly resembles the histological changes seen in human fibrostenotic CD.[203]

2.7.2.2. 2,4,6 trinitrobenzene sulfonic acid-induced colitis

Colitis can also be induced by intrarectal administration of TNBS in ethanol. Ethanol disrupts the epithelial barrier, allowing TNBS to elicit an delayed-type hypersensitivity reaction.[201] Repeated administration of TNBS over six weeks induces a chronic colitis with early disease characterized by a Th1 (mainly IL12 and IFNγ response), while in the chronic phase the Th2 response (IL13 and TGFβ) is predominant.[204] Eventually colorectal fibrosis develops with luminal stenosis and prestenotic dilatation and this is inflammation-dependent as NFκb inhibition has been shown to both suppress inflammation and development of fibrosis in this model.[204]

2.7.3. Immune-related models

The T cell transfer model is one of the best known immune-related IBD models. By injecting immune-deficient SCID mice with $CD4^{+}CD45^{RBhigh}$ T cells isolated from the spleens of Balbc mice, a wasting disease develops with severe, transmural inflammation. By selecting for $CD45^{RBhigh}$, naive T cells are isolated that evolve in colitogenic Th1/Th17 cells producing high amounts of TNFα and IFNγ Interestingly, injecting $CD45^{RBlow}$ cells, which contain Tregs can restore health in these mice. Alternatively, $CD4^{+}CD25^{-}CD62L^{+}$ T cells with CD25 being a marker for regulatory T cells and CD62L for naive cells can be used with similar results.[56,201 205]

A severe inflammatory colitis with infiltration of neutrophils, macrophages and lymphocytes is seen and with time a mild intestinal fibrosis develops.

2.7.4. Bacteria-induced models

Intestinal fibrosis develops in mice infected with *Salmonella Typhimurium* and is characterised by severe caecal inflammation resulting in fibrosis. Molecularly, TNFα, IL17 and IFNγ are upregulated as well as TGFβ and CTGF signalling. An important limitation of the model is that Salmonella does not elicit fibrosis in humans, limiting its relevance for the condition.[206]

Infecting streptomycin pre-treated mice with *enteroinvasive Escherichia coli* elicits a Th17 response, resulting in transmural inflammation and subsequent fibrosis.[207]

Lastly, intramural injection of peptidoglycan-polysaccharide into the intestinal wall induces a very local inflammation followed by bowel wall thickening and fibrosis with increased levels of TGFβ and IGF-1.[56,201]

2.7.5. Radiation-induced models

Exposure to therapeutic doses of radiation can induce a CD-like inflammation and fibrosis. Mainly applied in rats, one possible technique exists in externalizing a bowel segment from the abdominal cavity and irradiating it while covering the rest of the animal with a lead shield. Alternatively, a bilateral orchiectomy can be performed followed by the fixating a bowel segment in the scrotum allowing for irradiation without abdominal surgery. On a molecular level, fibrosis mainly develops because of prolonged upregulation of TGFβ and CTGF. The Rho/ROCK pathway also plays an important role in this model of fibrosis.[56,201]

2.7.6. Post-operative models of fibrosis

Postoperative recurrence of stenosis at the site of the anastomose occurs in about 70% of fibrostenotic CD patients undergoing surgery. Similarly, $IL10^{-/-}$ mice will develop small bowel fibrosis following Ileocaecal resection at the site of the anastomose and surrounding bowel segments, making this an ideal model to study this type of surgical recurrence. Interestingly, the recurrence does not develop in germ-free conditions, highlighting the importance of the microbiota in this model.[208]

Heterotopic transplantation of small bowel segments into the neck fold of rats is another post-operative fibrosis model. Crypt structures are lost very early after transplantation, followed by a dense leukocyte infiltration and eventually development of fibrosis.[177]

2.7.7. Genetic models of fibrosis

$IL10^{-/-}$ mice develop a spontaneous ileitis which is complicated by intestinal fibrosis with longstanding disease.[56,201] Adenovirus-induced overexpression of TGFβ results in intestinal collagen deposition, myofibroblast activation and fibrosis.[106]

Animal model	Method of fibrosis induction	Advantages	Disadvantages
SAMP1/Yit mice	- Spontaneous development of segmental inflammation and fibrosis - Genetic model	- Most representative CD model - Ileal fibrosis - Stricture development	- Difficult to breed
Chronic DSS	- Chemically induced - Repeated cycles of oral DSS - Chronic inflammation through disruption of epithelial barrier	- Relatively easy - Reproducible - Histological changes similar to fibrosis in human CD	-Fibrosis induction not representative for human CD - Colonic fibrosis - No stricture development
Chronic TNBS	- Chemically induced - Repeated cycles of rectal TNBS - Chronic inflammation through delayed hypersensitivity	- Relatively easy - Histological changes similar to fibrosis in human CD - Stricture development - Fibrosis induction representative for human CD	- Variable induction of fibrosis - Colonic fibrosis
T Cell transfer model	- Immune mediated - IP Injection of colitogenic Th1/ Th17 cells in immune deficient mice	- Representative action mode for human CD fibrosis	- Labour intensive - Mild colonic fibrosis - No stricture development
Salmonella model	- Bacteria induced - Severe caecal inflammation/fibrosis	- Allows for investigation of the role of microbiota	- Not representative for human CD - Colonic fibrosis - No stricture development
E. Coli model	- Bacteria induced - Th17 – induced inflammation	- Allows for investigation of the role of microbiota	- Not representative for human CD - Colonic fibrosis - No stricture development
PG induced model	- Bacteria induced - injection of peptidoglycans in the bowel	- Allows for investigation of the role of microbiota	- Not representative for human CD - Colonic fibrosis - No stricture development
Radiation induced model	- Selective irradiation of bowel segments - Rho/ROCK mediated	- CD-like inflammation and fibrosis - Ileal fibrosis	- Not representative for human CD - Labour intensive
Ileocaecal anastomosis in IL10-/- mice	- Postoperative fibrosis development - Genetically modified mice	- Allows for investigation of postoperative recurrences - Ileal fibrosis	- Difficult to breed - Postoperative mortality
Heterotopic bowel transplantation	- Small bowel transplantation into the neck of rats	- Ileal fibrosis	- Complicated technique - Not representative for CD fibrosis

Table 1 – Overview of different animal models in CD fibrosis.

3. Genetics of fibrostenotic Crohn's disease

3.1. Genetics of inflammatory bowel disease

Epidemiological studies dating from the 1980s, based on empirical observations of different prevalence rates between populations combined with unusually high incidence rates in certain population groups (e.g. Ashkenazi Jews) indicated that IBD is a genetically determined disease.[10,209] Familial aggregation and twin concordances studies provided further evidence but it was only in the mid 1990s that the first genetic regions associated with IBD were identified.[210,211] By means of linkage studies, the first IBD susceptibility region (named IBD1) was mapped to chromosome 16 and subsequently eight other linkage regions were identified (aptly named IBD2-9). These initial loci were typically very large, complicating the identification of causative genes.[10,212]

In 2001, the first IBD susceptibility gene (the pattern receptor Nucleotide-binding Oligomerization Domain-Containing 2 (*NOD2*)) located within the IBD1 locus was identified.[213,214] In the years to follow, other susceptibility genes were identified such as the organic cation transporter (*OCTN1-2*) gene on IBD5, *NOD1*, *TLR4* and the disks large homolog 5 (*DLG5*) gene, but it was only with the implementation of GWAS that the identification of associated genes accelerated drastically.[215 216 217,218] The first GWAS in CD was performed in a Japanese population and published in 2005[219], rapidly followed by other association studies in European populations and ulcerative colitis.[220 221 222-227 228 229] Later, major meta-analyses by the International consortia such as the International IBD Genetics Consortium (IIBDGC), further increased the power to identify associated loci and ultimately led to the discovery of 210 susceptibility genes.[230 231 232 233] Association studies using Immunochip data added another 6 loci and, whereas the initial GWAS were bases on Caucasian populations, recent trans-ethnic GWAS datat the current total to 242 genes associated with IBD.[234,235]

Discussing all of these individual risk alleles is beyond the scope of this work. However, two decades of genetic research have resulted in some important new insights in the disease biology of IBD.

3.1.1. IBD is a multifactorial, polygenic disorder

Although monogenic forms of IBD exist, especially in the pediatric population, they are extremely rare and the genetic risk in the majority of cases is based on a combination of several low risk, very commonly occurring variants (the so called common disease, common variant hypothesis).[236]

Indeed, most of the IBD associated susceptibility genes represent only minor risk increases generally not exceeding a relative risk of 30%.[13] The strongest genetic risk factor for IBD in general results from polymorphisms in the IL23 receptor (*IL23R*) gene and is associated with an odds ratio (OR) of 2.01. Specifically, for CD, *NOD2* variants represent the biggest risk increase (OR=3.01) while for UC SNPs in the human leukocyte antigen (HLA) locus and the *ADCY7* gene (adenylate cyclase 7, involved in conversion of ATP to cAMP) are most important with respective odds ratios of 1.44 and 2.19.[10,237] Most other risk variants identified in the GWAS studies represent a much lower risk, but are very common in the general population (**Figure 6**).

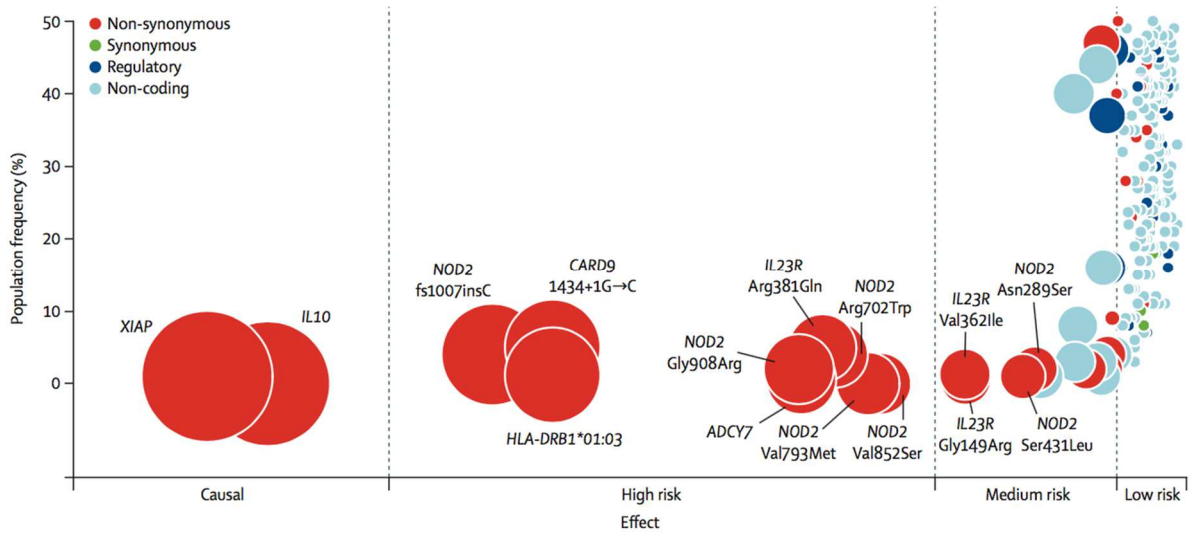

Figure 6 – Genetic risk in inflammatory bowel disease. The genetic risk in IBD consists of a combination of both rare and common variants. The most important genetic risk factors are plotted based on their minor allele frequency and disease risk. [Figure from Mirkov et al.[13]]

Despite the huge number of genetic risk variants identified, taken together these common variants only explain 26% of the heritability in CD and 19% in UC.[13] The entire IBD trait heritability, however, estimated from twin concordance studies, is thought to be around 75% for CD and 67% for UC.[238] Several reasons can explain this observed difference in heritability. First of all, other factors besides conventional genetics, such as epigenetic modifications, might account for a part of this missing heritability (discussed in 3.4). Secondly, the heritability rates in twin studies might be overestimated because of a dominance genetic effect, epistasis or shared environmental factors.[13] Additionally, many rare genetic variants can not be adequately detected by current GWAS.[239] Lastly, variants not commonly addressed by current GWAS analyses (e.g. genes located on the sex chromosomes) might play a role. Indeed, a variant in the *ARHGEF6* gene on chromosome X was only recently discovered by reanalysing the X-linked data from past GWAS studies.[240]

In the post-GWAS genetic research will have to focus on integrating insights coming from genomic research with data from other –omic fields such as transcriptomics, proteomics, epigenomics, metabolomics, microbiomics to get a more integrated view of IBD pathophysiology (the so-called IBD interactome).[241]

3.1.2. Genetics and pathways in IBD disease biology

The discovery of certain susceptibility loci has provided better understanding of the disease pathophysiology. For instance, the association with *NOD2* has illustrated the relative importance of the innate immunity in IBD, strengthened even more by other risks alleles in genes involved in innate mucosal defence such as *CARD9, TLR4* and *FCGR2A*.[25] The identification of the *ATG16L1* gene as a risk allele for CD (and later on the *IRGM* and *ATG4B* genes) on the other hand has opened up an entire new field of research by connecting the autophagy pathway to IBD.[188] Other risk variants have highlighted the importance of certain pathways within the adaptive immune system: for example the discovery of the *IL23R* susceptibility gene has not only given us a better understanding of the role of Th17 cells in IBD but even led to a new therapeutic agent (Ustekinumab).[242] The role of the epithelial barrier in IBD was emphasized as well by genetic findings: several risk factors identified in the GWAS studies are involved in barrier function (e.g. *CDH1* (cadherin-1, involved in epithelial cell junctions), *LAMB1* (laminin-1, involved in adhesion) and mucin-related genes (*MUC3A*, *MUC19*)). An overview of the different IBD disease pathways implicated by genetic research is given in **Figure 7**.[243]

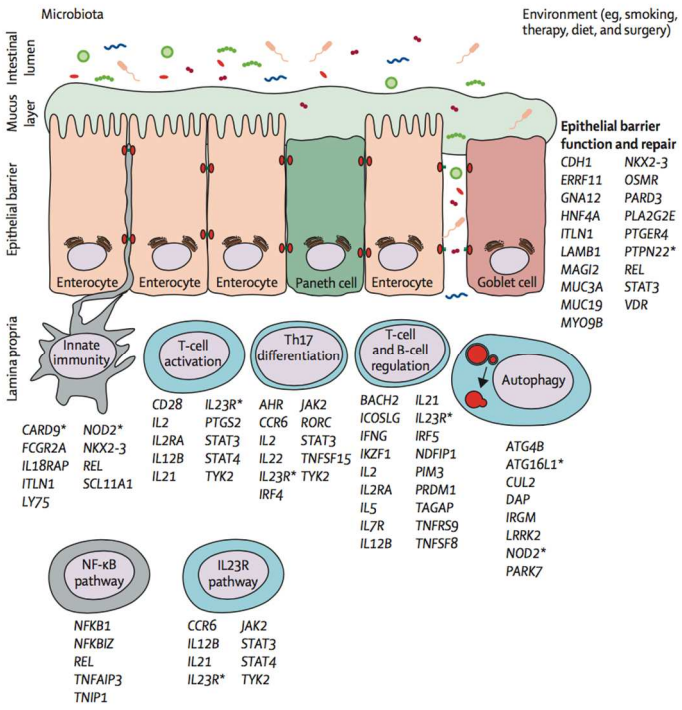

Figure 7 – Overview of disease pathways in inflammatory bowel disease identified by genetic association studies. Maior pathways identified by genetic studies are represented with their associated genes indicated. [From Mirkov et al.[13]]

3.1.3. Genetic resemblance between Crohn's disease, Ulcerative Colitis and other immune-related disorders

Although initially only a fraction of the disease loci identified in CD could be replicated in UC, a large meta-analysis combining several GWAS studies in 2011 showed that there is an important overlap between both clinical entities.[13,232] Approximately 67% of genetic variants (110 of the 163 at the time) were shared between both diseases, although there were large differences in effect size of many individual risk alleles between CD and UC. About 30 genes were considered CD specific as opposed to 23 UC-specific risk alleles. However, 81% of these disease-specific genes showed the same direction of effect-size in both phenotypes, leaving only a handful risk alleles that differ between CD and UC.[13] [10] [232] Genetic variants in the *NOD2* and *protein tyrosine phosphatase non-receptor type 22* (*PTPN22*) genes, for example, have protective effects in UC while being risk factors for CD.[10]

Interestingly, 66 of the risks alleles identified in IBD are shared with other immune-related disorders.[232] A cross-phenotype study examining different seronegative immune-related disorders (UC, CD, primary sclerosing cholangitis, psoriasis and ankylosing spondylitis), however, showed that each phenotype has a distinctive genetic profile despite the fact that they share a large number of susceptibility genes (244 loci in total).[244]

3.1.4. Monogenetic forms of inflammatory bowel disease

Monogenetic forms of IBD can sometimes give important insights into disease pathophysiology. Although over 50 monogenic forms of IBD have been identified, they still remain very rare despite the rising incidence of very-early onset IBD (VEO-IBD, defined as IBD occurring before the age of six). Approximately 14 per 100,000 children will be affected by VEO-IBD with an estimated incidence of 4.37 per 100,000 children. The exact fraction of these children that will have a monogenetic cause is unknown but is believed to be low.[245] In a case-series of 66 VEO-IBD patients, only 5 were identified as monogenic.[246] Well known examples of monogenic IBD are associated with mutations in the *IL10R* and X-linked inhibitor of apoptosis (*XIAP*) genes.

3.2. Genetic factors associated with fibrostenotic Crohn's disease

Several risk alleles for fibrostenotic CD have been put forward, however non have been linked casually. In this chapter a detailed overview of the most frequently associated genes is given.

3.2.1. Nucleotide-binding Oligomerization Domain-Containing 2 (NOD2)

The *NOD2* gene, a member of the caspase recruitment domain containing proteins, holds one of the strongest associations with fibrostenotic disease.[247]

NOD2/CARD15 functions as a cytosolic pattern recognition receptor involved in bacterial sensing and is in the intestine mainly expressed in epithelial cells, cells of the myeloid lineage (primarily in monocytes/macrophages, $CD40^+/CD86^+$ dendritic cells and stromal cells) and Paneth cells.[248,249] It functions as a receptor for muramyl dipeptide (MDP), a component of the cell wall found in both Gram-negative and Gram-positive bacteria. Upon binding of MDP, NOD2 interacts with *receptor-interacting serine-threonine kinase 2*, leading to the ubiquitination of the *NFκB essential modulator*, the key scaffolding protein associated with NFκB, allowing for its nuclear translocation and induction of inflammatory cytokine production (e.g. IL1β).[250]

The *NOD2* gene was first linked with CD in the early 2000s [213] and the association was subsequently confirmed in large genome wide association studies.[251,252] Although several other *NOD2* variants have been detected, three common single nucleotide polymorphisms (SNPs) represent the strongest association: two missense mutations, R702W in exon 4 (rs2066844) and G908R in exon 8 (rs2066845), and one frameshift mutation, Leu1007fsinsC (rs2066847), which truncates the protein with 30 amino acids.[247] Through genotype-phenotype studies, attempts have been made to associate these variants with disease behaviour, but a uniformly clear association with fibrostenotic disease has not been established. In 2004, Heresbach et al[253] were the first to report that the R702W acts as a strong predictor of fibrostenotic disease, irrespective of ileal location, but this could not be replicated by any of the subsequent studies. In a meta-analysis of 49 studies including 8,893 patients, NOD2 G908R was found to be the only variant associated with fibrostenosis[254], while other studies reported a strong association with the NOD2 Leu1007fsinsC variant.[255,256-258] In contrast, however, several large recent genotype-phenotype association studies did not find any relation with NOD2 variants after correcting for ileal disease.[259,260,261]

The lack of consistency in the data concerning *NOD2* and fibrostenotic disease is largely due to most analyses performed being subanalyses of larger trials using imperfect definitions of fibrostenotic disease. Indeed, most of the studies use the Montreal classification to identify fibrostenosis (Montreal B2) which lacks sensitivity, specificity and has a high inter observer disagreement rate.[262] Additionally, several studies did not correct for ileal location of the disease which is a known confounder for studies investigating fibrostenotic disease. One explanation might be the narrower lumen of the ileum

compared to the colon which results in a more rapid functionally significant stenosis. Alternatively, the inflammatory immune response in the ileum might be more severe than in the colon (resulting in more structural damage and consequently more fibrosis) due to the presence of the Peyer's patches, but this has not been clearly demonstrated.[259]

3.2.2. Autophagy-Related 16 Like-1 (ATG16L1)

ATG16L1 is an essential component of the autophagic pathway which in itself represents an important cellular homeostasis mechanism involved in both the innate and adaptive immune system.[263] The *ATG16L1* gene is located on the chromosome 2q37 and encodes for an adaptor protein involved in the elongation phase of the phagofore. Nine genetic variants have been linked to CD with the most common SNP being rs2241880, a missense variant resulting in a threonine-to-alanine substitution at nucleotide 300.[247] This association was first identified in 2007 by Hampe et al[264] and was subsequently confirmed in other European populations [265-267], but surprisingly not in any Asian meta-analyses.[263] Phenotypically, variants in *ATG16L1* were first linked with ileal disease location, irrespective of stricturing disease[268]. Two studies were able to link ATG16L1 T300A to fibrostenotic disease, one in an Australian adult [269] and one in a paediatric population.[270]. A recent study in a large European adult patient population, however, could not confirm this association.[47]

Structurally, the ATG16L1 protein consists of an N-terminal Atg5-binding region, an amino-terminal coiled-coil domain followed by seven tryptophan-aspartic acid (WD40) repeat domains. The rs2241880 variant is a nonsynonymous SNP changing adenosine to guanine in the WD40 repeat domain encoding a threonine to alanine substitution and making it more susceptible to caspase 3-dependent degradation upon starvation-induced metabolic stress, TNFα stimulation and infection with pathogenic bacteria resulting in an impaired autophagic response.[271] This diminished ability to form autophagosomic bodies interferes with normal bacterial clearance and induces a hyper inflammatory state with higher baseline IL1β and IL6 levels in epithelial specific *Atg16l1* deficient mice.[272] Moreover, Levin et al were able to shown that in patients carrying the T300A variant induction of anti-TNF-induced macrophages, that have an immunosuppressant function, was impaired.[273] Speculatively, this hyper inflammatory state might stimulate mesenchymal cells to produce excessive amounts of collagen.[247] Interestingly, there seems to be an important interaction between NOD2 and ATG16L1 as NOD2 can directly activate autophagy by interacting with ATG16L1.[263] Conversely, ATG16L1 is able to reduce pro-inflammatory NOD2 signalling in an autophagy-independent manner, an interaction that is impaired in patients with the T300A variant.[274]

3.2.3. Interleukin-23 Receptor (IL23R)

The *IL23R* gene encodes for one subunit of the heterodimeric receptor IL23 receptor (comprising IL23R and IL12Rβ1), and is found on the membrane of memory T cells, natural killer (NK) cells, monocytes and dendritic cells.[247] The pro-inflammatory IL23 in itself is a heterodimer build up out of a IL23p19 and a IL12p40 subunit, is secreted constitutively in the terminal ileum from macrophages, neutrophils, dendritic and epithelial cells and is required for maintenance and function of Th17 cells responsible for IL17 secretion.[242]

The *IL23R* gene itself is located on chromosome 1p31 and was first identified as a susceptibility gene by Duerr et al.[220] The most strongly associated SNP (rs11202926) in this study encodes an amino acid change from an arginine to a glutamine at position 381 and represents a reduced risk for IBD and other immune-related disorders such as spondylitis ankylosans and psoriasis.[242] In a German population-based study, a genotype-phenotype relation between fibrostenotic disease and another SNP (rs1004819) within the IL23R gene was found, in which homozygous carriers of the TT allele had an increased risk for ileal involvement and fibrostenosis compared to the wild-type CC allele although the association was not significant after Bonferroni correction.[275]

Other genes associated with fibrostenotic disease are listed in Table 1.

Gene	Polymorphism	Chr	Association	Mechanism	Population	Reference
CX3CR1	rs3732378 rs3732379	3	Ileal disease; fibrostenosis Fibrostenosis	Leukocyte adhesion	German (N=206) Caucasian (N=239)	Brandt et al[276,277] Sabate et al. [289]
TGFB	rs1800471	19	Fibrostenosis, faster progression to surgery	Increased serum TGFb levels	Australian (N=235)	Hume et al [278]
MAGI1	rs11924265	3p14	Fibrostenosis	Epithelial barrier dysfunction	Spanish (N=1296)	Alonso et al[279]
MMP-3	−1613 5T6T	11q22	Colonic disease, fibrostenosis	Elevated MMP-3 levels	Dutch (N=134)	Meijer et al[280]

JAK2	rs10758669		Ileal disease; fibrostenosis	Epithelial barrier dysfunction	Caucasian (N=1598)	Cleynen et al[47]
FUT2	rs601338	19q13	Fibrostenosis	Microbiota disturbance	Belgian (N=647)	Forni et al[281]
IL12B	rs1363670		Fibrostenosis in ileal CD	Unknown	Belgian (N=875)	Henckaerts et al[282]

Table 2 - Genes associated with fibrostenotic disease

3.2.3. Cautionary notes on genetic associations in fibrostenotic CD

None of the studies described above have been able to identify a single uniform variant that is independently associated with fibrostenotic CD. One of the main reasons for this lack of reproducibility is the lack of power in the individual studies. Most of the data indeed comes from subanalyses of larger trials, leading to a relatively small number of included patients in these studies.

Secondly, the definition used to identify fibrostenotic CD patients varies considerably between the different studies and most of them use the clinically based Montreal classification to define stricturing disease. Although frequently used in large population studies, the Montreal classification has known issues of low sensitivity, low specificity and a high inter-observer disagreement for identifying fibrostenosis, making it far from ideal to use in phenotype-genotyping studies where a strict definition of the phenotype is crucial.[262]

Thirdly, many of the studies investigating genetic associations in fibrostenotic CD have not taken into account possible confounding factors. For example, disease location is an important driving force in disease behaviour over time and studies not correcting for ileal disease location should be interpreted with caution. Other known confounders for stenotic disease are smoking status and medication use (especially NSAIDs).[283]

A last note of consideration is disease duration. The speed with which fibrostenosis develops differs strongly between patients. At diagnosis, only 10% of patients presents with stenotic complications and it steadily rises to 30% after 10 years.[50,284] Why some patients develop fibrostenotic disease more early then others is unknown, but a genetic factor might partly explain the differences seen. Early developers of fibrostenosis might represent an entirely different population with a stronger genetic

risk. To date no association study, including those performed by the IIBDGC, have taken into account time to development of fibrosis.[260]

3.3. Epigenetics

Although there has been tremendous progress in our understanding of the heritability of IBD thanks to the recent advances in GWAS studies, all of the identified genetic factors and susceptibility loci combined only account for 13.6% of disease variability in CD and explain only 26% of the observed genetic risk. These numbers are even worse in the case of UC where only 7.5% of disease variability is explained.[14,285] This first of all puts a critical note to the genetic findings to date and otherwise emphasises the importance of environmental factors in the pathogenesis of IBD. One mechanism by which the environment could modify the heritable risk of IBD is through the process of epigenetics. In this chapter the basic principles of epigenetics, epigenetic changes and their possible role in IBD pathogenesis and more specifically in intestinal fibrosis will be addressed.

3.3.1. Influence of epigenetics in inflammatory bowel diseases

3.3.2.1. DNA methylation and IBD

First suggestions that differences in DNA methylation patterns might play a role of importance in IBD comes from the identification of *DNTM3a* and *b*, key enzymes in DNA methylation, as susceptibility genes for CD.[231] Initial so-called epigenome-wide methylation association studies (EWAS) focussed on whole-blood DNA and identified differently methylated regions (DMR) in over 50 genes between patients with ileal CD and normal controls. Many of the identified genes in this study play an important role in immune-regulation (*MAPK, FASLG, S100A13, RPIK3, IL21R*) including the Th17 pathway.[286] Epigenetic markers are highly tissue- and even cell-specific, making these whole-blood DNA results difficult to interpret.

Further studies have focused more on tissue- and cell type specific epigenetic changes. Harris et al showed hypermethylation of the *TEPP* gene (testis, prostate and placenta-expressed protein) specifically in PBMCs isolated from a pediatric CD population.[287] The functional consequences of this finding, however, remain unknown. Other DMRs identified in PBMCs of IBD patients include *TRIM39-RPP2* (involved in the type I interferon pathway) and *TRAF6* (involved in TNFα signal transduction).[288] Others have investigated DNA methylation patterns in colonic tissue from IBD discordant monozygotic twins (thus correcting for genetic differences) and found DRMs in over 61 genes including several immune-regulating ones (e.g. *CFI, SPINK4, THY1/CD90*).[289] Kang et al identified additional DRMs in *FAM217B*, *KIAA164* and *RIBC2*, although functional consequences remain unknown.[290]

To further overcome the heterogeneity of cell types found in PBMCs and tissue biopsies, some studies have studied DNA methylation in specific cell populations. Ventham et al for instance identified several new IBD-associated DMRs including *VMP-1* (vaculole-membrane protein 1) in which hypomethylation of the primary transcription site of microRNA-21 (miR21), a known pro-inflammatory structure in IBD, was found. Other identified regions include *ITGB2* (integrin subunit beta 2, involved in leukocyte trafficking), *RPS6KA2* (involved in the PI3K/Akt/mTOR pathway and autophagy) and *TXK* (a member of the Tec family of non-tyrosine kinase receptors). Interestingly, they found these DNA methylation patterns to vary between different leukocyte subsets. For example, hypermethylation of *TXK* only occurred in CD8+ T cells, where it is necessary for interferon gamma production.[291] DNA methylation patterns in intestinal epithelial cells were found to be different between active and quiescent UC in a study by Cooke et al, including DOK2 (involved in IL4-mediated proliferation), Tap1 (MHCI transport molecule) and members of the TNF family (TNFSF4 and – 12).[292]

3.3.2.2. Histone modifications in IBD

Although increased histone acetylation of H4 is present in inflamed intestinal biopsies of CD patients, at present only indirect evidence coming from experimental studies using histone deacetylase inhibitors (HDACi) links histone modification to IBD.[293] Butyrate, a short-chain fatty acid produced by the intestinal microbiota, is a naturally occurring HDAC inhibitor, has an anti-inflammatory effects in murine models of IBD and has even shown potential in the treatment of IBD patients.[14,294] Although butyrate increases both NOD2 expression and the production of intestinal alkaline phosphatase (necessary for detoxification of LPS) by increasing histone acetylation, butyrate has several other anti-inflammatory modes of action (e.g. inhibition of NFκB) which are histone independent.[295,296] Other HADC inhibitors that also have shown potential in murine colitis, are similarly known to work via other mechanisms than epigenetic modification, making it difficult to apprehend the exact role of histone modification in IBD.[297,298]

3.3.2.3. Interference by microRNAs

MicroRNAs play an important role in the gut homeostasis. Mice deficient in Dicer1, an enzyme involved in miR processing, show spontaneous intestinal inflammation and a disturbed epithelial barrier.[299] In human IBD, miR levels have been shown to vary between patients and healthy controls. miR-192, which controls the expression of macrophage inhibitory peptide 2α in intestinal epithelial cells, is lowered in colonic tissues of UC patients,[300] while miR-150 expression (controlling c-Myb expression involved in apoptosis) is increased.[301] Levels of the pro-inflammatory miR-21 and -155, are increased in both active UC and CD colitis (but not CD ileitis) patients. [302,303] Elevated concentrations of miR-196 have been found in the inflamed epithelium of CD patients and are associated with a

defective IRGM-mediated autophagic respons.[304]

3.3.3. Role of epigenetics in intestinal fibrosis

Much of the evidence for epigenetic modifications as a player in the pathophysiology of intestinal fibrosis comes from other organ systems as few studies have directly investigated effects of epigenetics on fibrostenotic disease.[285]

3.3.3.1. DNA methylation

Several EWAS studies have shown differential DNA methylation patterns in **idiopathic pulmonary fibrosis** (IPF) patients as opposed to healthy controls. [305,306] Most notably, changes in DNA methylation of the αSMA promotor gene were found in pulmonary fibroblasts isolated from IPF patients.[307] In **renal fibrosis**, a set of fibrosis-related genes including several types of collagens, was differentially methylated between patients and controls.[308] In a rat model of **cardiac fibrosis**, hypermethylation of several genes was found alongside an increased expression of DNMT-1 and -3b.[309]

In fibrostenotic CD, Sadler and colleagues compared DNA methylation profiles between intestinal fibroblasts isolated from the colon of fibrostenotic CD patients and normal fibroblasts and integrated them with transcriptomic data. They identified three hypermethylated regions in stenotic fibroblasts (wingless-type mouse mammary tumor virus integration site family, member 2B (WNT2B), prostacyclin synthase and prostaglandin D2 synthase) which resulted in a reduced transcription of these genes.[310] Another possible link might be through differential methylation of the VMP1 gene which has been frequently reported on in general IBD EWAS studies.[291] As previously discussed, the miR-21 gene lies within the VMP1 locus and increased levels are present in intestinal fibroblasts of patients with fibrostenotic CD. Functionally, elevated levels of miR-21 induce a sustained TGFβ response which lead to an increased deposition of collagen and ECM.[311]

3.3.3.2. Histone modification

Histone hyperacetylation has been linked to **pulmonary fibrosis** in a number of studies. [312-314] Hyperacetylation of H3 specifically results in an increased secretion of collagen and MMP-1 by pulmonary fibroblasts and is involved in increased cyclooxygenase-2 and Thy-1 cell antigen expression both of which are integral in the pulmonary fibrosis development.[313,314] A similar process increases expression of pro-fibrotic genes such as collagen, TGFβ1 and αSMA in **hepatic stellate cells**, while elevated p300 acetyl transferase activity leads to an increased collagen production in **systemic sclerosis**.[315,316]

Specifically, in CD, hyperacetylation of histone H3 and H4 has been found, however no direct studies linking histone modification to fibrostenotic disease are available. Indirectly, however, Sadler et al showed that in endoMT, hyperacetylation of H4 in the *COL1A2* promotor region, induced by IL1β, TGFβ and TNFα, results in an increased transcription and secretion of collagen.[317]

3.3.3.3. MicroRNA interference

MicroRNA interference plays a role in fibrosis throughout the body, including lungs, heart, kidneys and intestine.[285] The microRNA miR-21 gene is located on chromosome 17 within the VMP-1 gene and its transcription product has a consistently pro-fibrotic role throughout different organ systems. miR-21 regulates transcription of Sprouty-homolog 1 (Spry-1), phosphatase and tensin homologue (PTEN), peroxisome proliferator-activated receptor α (PPAR-α), signal transducer and activator of transcription-3 (Smad3) and Smad7, all of which are known cellular regulators of fibrosis (see above).[311,318-320] miR-29a,b,c on the other hand has a decidedly anti-fibrotic role and suppresses transcription of collagen, MMPs and Spry-1 in fibroblasts isolated from the intestine, skin, heart and kidney.[311,321-323] Lastly, the miR17/92 cluster represent an evolutionary highly conserved group of 6 microRNAs located on chromosome 13.[324] Several of these miR's play a role in fibrosis by targeting key molecules involved in its pathophysiology including TGFβ (miR-17; miR-19a,b); collagen I (miR-18a,b; miR-19a,b) and MMPs (miR-17; miR-18a,b; miR-19a,b).[325]

4. (Bio)markers of fibrosis

At this moment, no specific and adequate marker for prediction or diagnosis of intestinal fibrosis is available. Finding such a marker is important for several reasons. First of all, clinicians are often faced with the difficulty of determining whether a stricture is mainly fibrotic (and is amendable only by surgery or anti-fibrotic therapy) or has an important inflammatory component (and can thus be treated by anti-inflammatory therapy). Secondly, progress in finding new anti-fibrotic therapies is hampered by the lack of a good fibrosis marker as it makes constructing clinical trials difficult. The ideal fibrosis marker should detect fibrosis in an early stage, be both predictive and responsive to anti-fibrotic therapy and be able to signal non-responsiveness to anti-inflammatory therapy. In this chapter, currently available markers of intestinal fibrosis will be discussed alongside interesting markers identified in other fibrotic diseases that might be usable in the gut.

4.1. Clinical risk factors

The best-established predictors for developing fibrostenosing disease are purely clinical. Young age at CD diagnosis, the need for corticosteroids during the first presentation, perianal disease location, elevated serum CRP levels and the presence of deep colonic ulcers on endoscopy are all associated with a higher chance of a complicated disease course. Of note, these factors are not specific for development of fibrostenosis but more general predictors of a disabling disease course.[326,327]

4.2. Genetic markers

Genetic polymorphisms are ideal biomarkers as they are stable, present before disease onset and are unaffected by the disease course. Although several genes have been associated with intestinal fibrosis (see chapter 3), many of them exhibit an important bias with disease location and complicated disease in general. Moreover, population prevalence of these markers is generally low and penetrance is incomplete, making them unusable in clinical practise.[326,328]

4.3. Blood biomarkers of intestinal fibrosis

4.3.1. MicroRNA

miRs are the best studied form of epigenetic modifications and circulating levels of some miRs are increased in fibrostenosing CD. For example, serum levels of miR200b but not miR200a were found to be increased in patients with a stricturing phenotype.[329] miR29a, miR19a-3p and miR19b-3p serum levels, on the other hand, were lower in fibrostenosing CD and this irrespective of ileal disease location or disease duration.[322,330]

4.3.2. ECM products

Serum levels of N-terminal propeptide of collagen type III (PIIINP) are increased in fibrostenotic CD patients and levels dropped significantly after surgical intervention.[331] However, another study did not find any significant differences in PIIINP levels between CD patients and controls.[332] Although TIMP-1 levels are increased in the mucosa overlying CD strictures, circulating levels did not differ between fibrostenotic and inflammatory patients.[333] The elevated liver fibrosis (ELF) test, which combines circulating levels hyaluronic acid, TIMP-1 and PIIINP discriminates between stricturing and non-stricturing CD phenotypes.[328] Fibronectin levels are similarly increased in fibrostenotic CD and drop in the postoperative period, but were not predictive of stricture recurrence.[334]

4.3.3. Growth factors

Serum levels of several growth factors are elevated in CD patients, including FGF, human chitinase 3-like 1 (YKL-40) and PDGF. Of these, only FGF has been shown to discriminate between fibrostenosing and inflammatory phenotypes. Moreover, FGF levels correlate with bowel wall tickness.[118] Studies with YKL-40, however, which is released by activated macrophages and neutrophils and promotes (myo)fibroblast activation have produced contradicting results. [335,336] No association has been found between PDGF levels and fibrostenosis.[328]

4.3.4. Antimicrobial antibodies

Several antibodies against luminal microbiota are raised in the serum of CD patients and correlate with complicated disease. Anti-saccharomyces antibodies (ASCA) have a sensitivity of 70% and a specificity of 48% for predicting fibrostenosing or penetrating disease and are associated with an increased risk for surgery.[337] Anti-chitobioside carbohydrate IgA antibiodies (ACCA) have a higher specificity for fibrostenosis (70%) but a lower sensitivity (43%) than ASCAs.[338] In paediatric CD, seropositivity for anti-CBir1 (anti-flagellin antibodies), anti-OmpC (anti-E Coli outer membrane protein C antibodies), antibodies against *Pseudomans fluorescens* (anti-I2) and ASCA were predictive of developing a stricturing or penetrating phenotype.[339] However, a recent study did not find any association between these anti-glycan antibodies and disease phenotype.[340] Overall antimicrobial antibodies seem able to predict a complicated disease course, but are currently not useful to discriminate between stricturing and non-stricturing disease phenotypes.

4.3.5. Promising blood biomarkers for identifying fibrosis in other organ systems

Circulating fibrocytes have been proposed as a biomarker in IPF.[341] In intestinal fibrosis no data are available. The number of circulating fibrocytes, however, has been shown to be increased in the blood of CD patients.[342]

Proteomic analysis or examining the entire set of expressed proteins at a certain time by mass spectometry has been used successfully to predict different stages of liver fibrosis in hepatitis B infected patients and identified several differently expressed proteins including transferrin, alcohol dehydrogenase and annexin-4.[343] A similar study in methotrexate-induced hepatic fibrosis identified serotransferrin, haptoglobin and N-cadherin as being associated with liver fibrosis.[326] In IPF, a screening of 92 candidate proteins showed 5 of them (MMP-7, ICAM-1, IL8, VCAM-1, S100A12) to correlate with disease progression and mortality.[344] No studies are currently available in intestinal fibrosis.

In an exploratory study by Higgins et al, serum samples of 28 fibrostenosing CD patients scheduled for surgery were collected before surgery and at two time points after surgery (1 and 3 months). **Glycoproteomic analysis** (characterizing proteins containing carbohydrates chains as a posttranslational modification) identified two biomarkers: hepatic growth factor α and cartilage oligometrix matrix protein (COMP). Large scale, prospective studies are necessary to confirm these results.[345] In chronic hepatitis C patients, glycoproteomics identified a new biomarker for estimating liver fibrosis. LecT-Hepa, a fibrosis-related glyco-alteration of the serum alpha 1-acid glycoprotein, outperformed other non-invasive fibrosis tests in this study and correlated well with the degree of fibrosis. [346]

Metabolomics or analysing the chemical metabolites of cellular processes have been used in patients with chronic hepatitis C and found a distinct serum metabolic bioprofile to be associated with advanced fibrosis.[347] Similarly, a study in systemic sclerosis (SS) patients showed an increase in acetate, lactate, alanine and lipoprotein levels in patients who had concomitant pulmonary hypertension, an important prognostic marker of SS, compared to SS patients who did not have this complication.[348] No trials have been performed in fibrostenosing CD.

4.4. Discriminative imaging techniques

Imaging techniques have traditionally been used to try and discriminate between inflammatory and fibrotic stenosis. The absence of inflammatory signs on CT/MRI enlarges the chance of a purely fibrotic stenosis, however it is not a measure for the severity of the fibrosis nor does it have prognostic value

in predicting response to anti-inflammatory therapy.[345] Several novel imaging techniques have become available and hold promise in outperforming CT/MRI in identifying fibrosis.

Fibrotic strictures were found to display a delayed gadolinium enhancement on MRI and although correlation with the histological degree of fibrosis was generally poor, using an empirical cut-off of 24% enhancement between 70 sec and 7 min after contrast injection was able to discriminate between severe and non-severe fibrosis with a sensitivity of 94% and a specificity of 89%.[349]

Magnetization transfer (MT)-MRI is a MRI sequence that measures the magnetic spin transfer of hydrogen atoms in free water relatively to those bound to macromolecules such as collagen. This technique was able to adequately discriminate between rats in an acute TNBS model (with intestinal inflammation) and rats in a chronic TNBS model (that have developed intestinal fibrosis). MT-MRI has also been shown to correlate well with the collagen content in the bowel wall. Combining MT-MRI with T2 weighted images (T2/MT-MRI) can discriminate between inflammation and fibrosis with an area under the curve of 0.98.

Ultrasound elastography uses the ratio between an external compressive force and the resulting tissue compression to measure tissue stiffness. Practically, the ultrasound probe is used to identify the stricture, applied to compress the tissue and used to measure the change in thickness during compression.[345] It has been successfully used in animal models to differentiate between inflamed and fibrotic tissues and recently has been validated in fibrostenosing CD patients.

Frequently used for assessing liver fibrosis, shear wave elastography sends a pulse wave into the tissue generating vibrations that travel through it as a shear wave. Stiffer tissue will increase the velocity with which the wave travels, while softer tissue will transmit it more slowly.[350] In animal models this technique is able to differentiate between inflamed and fibrotic tissue and has been applied with success in resected CD strictures.[349]

In PET/CT, 18-fluoro-deoxyglucose (18-FDG) is used to measure glucose metabolism as a surrogate for inflammation. In a pilot trial of 17 CD patients 18-FDG PET could not accurately predict in which patients surgery could be avoided. Theoretically, it is possible to combine 18-FDG-PET with a labelled hydroxyproline that is incorporated into collagen for more accurate imaging of fibrostenosis, however this technique is prohibitively expensive.[345,349]

5. Current management of fibrostenotic Crohn's disease

At this moment there are no specific anti-fibrotic therapies available for fibrostenotic CD. Current management is based on control of any residual inflammation, followed if necessary by endoscopic dilatation or resection of the affected bowel segment. When fibrostenotic disease is suspected, cross-sectional imaging by CT or MRI is necessary to 1) confirm the diagnosis, 2) localise and visualise the extent of the disease 3) assess any residual inflammation that might be amendable by anti-inflammatory therapy.

4.1. Medical therapy

Medical therapy mainly consists of controlling the inflammation that is present to decrease bowel wall oedema, which might reduce bowel wall thickness and alleviate obstructive symptoms.[50] However, evidence supporting this approach is limited. In an open label trial of 26 CD patients with acute small bowel obstruction, corticosteroids relieved symptoms in all but one patient. However, 70% of patients relapsed and although most responded to retreatment with corticosteroids, eventually 46% failed medical therapy and required surgical intervention.[351] In the case of steroid-refractory patients, TNF antagonists can be successfully tried. In a prospective, multicentre, observational cohort study testing adalimumab in 97 patients with small bowel obstruction, 64% had not required corticosteroids, endoscopic dilatation or bowel resection at week 24. Of these, 47% still were intervention free at 4 years after adalimumab initiation.[352] No data about the efficacy of other anti-inflammatory agents for treating fibrostenosing disease are available.[50] When medical therapy fails, endoscopic treatment or surgery should be considered.

4.2. Endoscopic approaches

Several endoscopic options are available for treating fibrostenosis including endoscopic balloon dilatation (EBD), intralesional injection of corticosteroids or TNF antagonists and placing of a stent.

Indications for EBD are strictures within reach of endoscopy, < 5 cm in length, not-angulated and without any local complications (such as fistulisation or abscesses). In a retrospective analysis of 34 studies, EBD was successful in 89,9% of cases with clinical response in 79,5% and limited complications (2,6%). In a follow-up time of 40 months, 48,6% had recurring symptoms requiring surgical intervention in 30,2%.[353] Post-dilatation injection of corticosteroids appears to have no additional benefit according to a recent systematic review.[353] Although corticosteroid injection prolonged the time to redilatation in a paediatric population, in a randomised controlled trial in adult CD patients the reverse was observed.[354,355] Several case studies investigated the local injection of TNF antagonists, but both are currently not recommended in clinical practise.[356,357]

Retrospective evidence suggests a benefit of upgrading medical therapy after EBD, especially when there is severe inflammation present. In a retrospective study examining 54 CD patients that underwent EBD for an anastomotic stricture, upgrade of therapy to a TNF antagonist combined with an immunosuppressant was associated with a reduced risk of re-intervention (HR 0.23).[358] The risk of restenosis was highest when severe inflammation (Rutgeerts i4) was present at the site of anastomosis. However, serial dilatations of strictures are feasible and do not seem to impair outcomes or increase the risk of complications, making it an acceptable strategy in clinical practise.[359]

Metallic stent placing has been used successfully in colonic malignities and although the initial success rate in CD strictures approximates 100%, complications such as fistulisation and stent migration appear in about two third of patients.[360] Biodegradable stents that resolve within 12 weeks seem a promising alternative, but controlled studies are lacking.[361] Other interventional endoscopic techniques include using a sphincterotome or needle knife to carve the stricture have theoretical advantages of reducing stricture recurrence but at the cost of a higher risk of complications. Case series have been published, but as no head-to-head trials are available these techniques are not recommended in clinical practise.[50,52]

4.3. Surgery

When a stricture is too long (> 5 cm), dilatation is technically not feasible or is located ileocaecally, surgery is considered superior to medical therapy.[50] Strictures without local complications are eligible for stricturoplasty, which has the advantage of preserving bowel length and avoiding anastomotic complications. Different types of procedures are available: Heineke-Mikulicz for short segments (< 10 cm), Finney for intermediate length strictures (10-25 cm) and the isoperistaltic Michelassi procedure that is more suitable for longer strictures (> 25 cm) or multiple strictures in close proximity.[52] For colonic strictures, resection is always recommended considering the risk of an underlying malignancy.[50]

4.4. Anti-fibrotic therapy

Despite the considerable medical need, no direct anti-fibrotic therapies are currently available for treating intestinal fibrosis.

Surprisingly, in other organ systems specific anti-fibrotic agents have been available for years. In pulmonary fibrosis for instance, pirfenidone, an oral pyridone derivative that is thought to interfere with TGFβ signalling, has been FDA approved and improves disease progression and survival in IPF

patients. Nintedanib, a multikinase inhibitor, has shown similar results. Of note, all of these trials used clinical intermediate endpoints and not histopathology to prove anti-fibrotic efficacy. In cardiac fibrosis, ACE inhibitors, angiotensin-receptor blockers and mineralocorticosteroid-receptor antagonists have secondarily shown to reduce fibrosis on MRI aside from their benefits on cardiac function.[57] Particularly in liver disease many agents have proven to reduce and even reverse fibrosis: for instance HCV eradication, HBV anti-viral therapy, corticosteroid therapy for auto-immune hepatitis, treatment of biliary obstruction, alcohol cessation and phlebotomy in hemochromatosis all have shown beneficial effects on fibrosis and clinical outcomes.

In IBD-related fibrosis, several preclinical agents have been tested, however none have progressed to the clinical trial stage (**Table 2**). Most of these agents act by suppressing TGFβ signalling. However, total blocking of TGFβ signalling raises safety concerns as TGFβ has important anti-inflammatory effects as well. Indeed, TGFβ is the a potent natural immunosuppressor and complete abrogation of its function could result in spontaneous activation of B and T cells and suppression of regulatory T cell ($CD4^+CD25^+$) function ultimately leading to an increased inflammatory and autoimmunity response.[362] Novel, directly acting anti-fibrotic strategies are thus necessary before considering taking anti-fibrotic therapy into clinical trial in CD patients.

Agent	Class	Preclinical model	Principal mode of action	Reference
GED-0507-34	PPARγ agonist	Chronic DSS	Counteracts TGFβ/SMAD	[142]
Captopril	ACE inhibitor	Chronic TNBS	Counteracts TGFβ/SMAD	[139]
Enalaprilat	ACE inhibitor	Chronic DSS	Counteracts TGFβ/SMAD	[363]
Losartan	ARB	Chronic TNBS	Counteracts TGFβ/SMAD	[364]
Glutamine	Amino acid	Chronic TNBS	Counteracts TGFβ/SMAD	[365]
HSc025	YB-1 agonist	Chronic TNBS	Counteracts TGFβ/SMAD	[366]
Cilengitide	αvβ3 integrin antagonist	Chronic TNBS	Interferes with latent TGFβ activation	[367]
Simvastatine	Statine	Chronic TNBS	Promotes fibroblast apoptosis	[368]

Reversatrol	Phytoalexin	PG-PS model	Counteracts TGFβ/SMAD	[369]
TL1A inhibitor	Monoclonal antibody	Chronic TNBS	Counteracts TGFβ/SMAD Promotes fibroblast apoptosis	[370]
CALY-001	Anti-MMP9 monoclonal antibody	Heterotopic xenograft model	Counteracts MMP9	[174]

Table 3 – Overview of different preclinical agents tested in intestinal fibrosis

III. RHO KINASES IN INFLAMMATORY BOWEL DISEASE

Rho-associated coiled-coil forming protein kinase (ROCK) are a family of serine/threonine kinases involved in cytoskeleton formation and have a central role in fibrogenesis, making them ideal targets for anti-fibrotic therapy. Systemic inhibition of ROCK, however, is associated with severe cardiovascular side-effects, limiting their applicability. In this section, structure and function of the Rho kinase and their role in both inflammatory and fibrotic diseases will be discussed.

1. Molecular structure and physiological function of Rho kinases

ROCKs are 160kDa protein serine/threonine kinases consisting of a C-terminal RhoA binding domain and a N-terminal kinase domain. Two isoforms exist (ROCK1 and ROCK2) that share over 65% of their overal amino acid sequence with the highest similarity in their kinase domain (92%) and are expressed both in human and murine tissues. ROCK1 is ubiquitiously expressed, while ROCK2 is more selectively present in brain and muscle tissue, especially smooth muscle. In their inactive form, the C-terminal and RhoA binding domain bind the kinase domain forming an auto-inhibitoryloop. (**Figure 8**) activated, GTP-bound RhoA binds the RhoA-binding domain and induces a conformational change freeing the kinase domain. Substrates for phosphorylation by ROCK include the non-muscle myosin light chain II (MLC), MLC phosphatase (MLCP), Lin11, Isl1 and Mec3 kinase (LIMK), adducin and the ezrin-radixin-moesin (ERM) kinases. ROCK phosphorylates MLC at the same serine residue as the MLC kinase, enabling myosin binding to actin filaments that is required for contractile function. By inhibiting MLCP function, ROCK activation additionally raises phosphorylated MLC levels. Phosphorylation of the LIM kinases leads to deactivation of cofilin, an actin depolymerisation protein, ultimately leading to an increase in actin filaments. ROCK-mediated phosphorylation of the ERM proteins disrupts their head-to-tail, inactive formation allowing for actin cytoskeletal reorganisation. Taken together, ROCK activation ultimately leads to increased assembly of actin fibers and enhanced cytoskeletal contractility activating several inflammation and pro-fibrotic pathways in the process . [371-373]

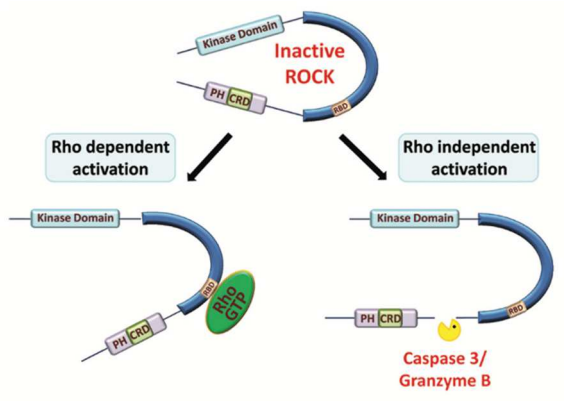

Figure 8 – Rho kinase activation. In the inactive state, the carboxy-terminal domain acts as an auto-inhibitory region. Binding of Rho-GTP to the Rho binding domain (RBD) induces a conformational change freeing the kinase domain. Alternatively, the C terminal domain can be cleaved by caspase 3 or Granzyme B, activating the Rho kinase. [From Julian et al.[372]]

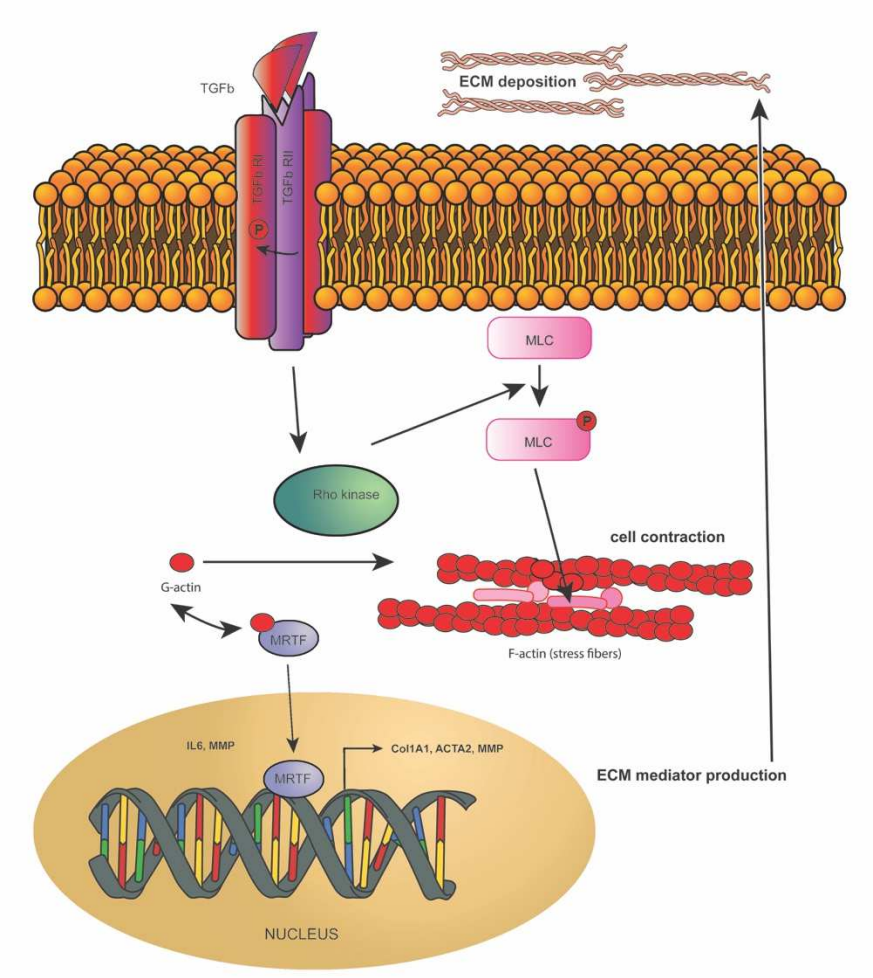

Figure 9 – Processes regulated by the Rho kinase pathway. Upon stimulation by growth factors (GF), TGFβ, mechanical stimuli or G protein-coupled receptor (GPCR) ligands, RhoA is activated by guanine nucleotide exchange factors (GEFs). Active RhoA activates ROCKs leading to increased actin polymerisation and release of the myocardin-related transcription factors (MRTF-A/B). MRTF-A/B will translocate to the nucleus and promotes transcription of serum-response factor (SRF) induced profibrotic genes (e.g. ACTA2).

2. Rho kinases in inflammation

Several lines of evidence suggest an important role of ROCK and ROCK signaling in inflammatory disorders. In a genetic association study in a Turkish population, polymorphisms in the ROCK2 gene (rs35768389 and rs1515219) were found to be associated with Behçet's disease, a multisystemic vasculitis disorder, and ROCK2 mRNA expression was increased in the peripheral blood of these patients.[374] Additionally, 60% of patients with systemic lupus erythematosus show an increased leukocyte ROCK activity and ROCK inhibition has shown to be beneficial in experimental models of both lupus and rheumatoid arthritis.[375-377] In murine auto-immune encephalomyelitis, an experimental model for multiple sclerosis, ROCK inhibitors can delay disease onset and severity, while in a LPS-induced murine model of renal failure treatment with ROCK inhibitors alleviated inflammation.[378] [379]

Specifically in IBD, Segain et al showed increased ROCK activity in the inflamed mucosa of IBD patients and using a non-selective ROCK inhibitor (Y27632) significantly reduced colonic inflammation in a TNBS-induced colitis model. Mechanistically they could show that ROCK inhibition decreased pro-inflammatory cytokine release from PBMCs by downregulation of NFκB signaling.[380]

ROCK and ROCK based signaling play an important role in the function of several immune cells. ROCK inhibition hampers chemokine-induced **T cell** polarization and migration of both **B and T cells** by interfering with the cytoskeletal reorganization necessary for these processes.[379,381] Similarly, **dendritic cell** (DC) morphology is profoundly altered by ROCK inhibitors, resulting in dendrite retraction, affecting DC-T cell interactions and impairing antigen presentation. In **macrophages**, ROCK inhibitors disrupt the shift towards an M1 phenotype and downregulate production of pro-inflammatory cytokines as IL1β and TNFα by interfering with NFκB signaling.[379] Taken together, these data suggest a possible therapeutic role of ROCK inhibitors in inflammation. Of note, however, in most of these experiments non-selective ROCK inhibitors were used (see section 4).

3. Rho kinases and their role in fibrotic diseases

3.1. ROCK and pro-fibrotic signaling pathways

ROCK signaling is activated by numerous pro-fibrotic signals including increased matrix stiffness, thrombin and TGFβ, leading to actin fiber assembly which is essential for (myo)fibroblasts migration and activation.

Increased assembly of actin fibers ("stress fibers") involves polymerization of the globular (G)-actin monomer into filamentous (F) actin fibers. G-actin sequesters the myocardin-related transcription factors (MRTF) A and B to the cytosol and polymerization liberates MRTF allowing it to translocated to the nucleus and ultimately result in the transcription of serum response element containing genes such as TGFβ, CTGF, αSMA, collagen and fibronectin. Additionally, actin polymerization has been shown to inhibit Lats 1/2 kinases resulting in a dephosphorylation of the Yes-associated protein (YAP) and its transcriptional coactivator with PDZ domain (TAZ). Phosphorylation sequesters YAP/TAZ in the cytosol through binding to the 14-3-3 proteins. Dephosphorylised YAP/TAZ translocates to the nucleus and activates transcription factors of the TEA domain family (TEAD) resulting in the transcription of CTGF and other profibrotic factors.[371]

ROCK signaling is not only activated by TGFβ but is also essential for its pro-fibrotic actions. First of all, activation of latent TGFβ by av integrins requires ROCK-dependent cytoskeletal reorganization.[371] Secondly, although ROCK is not required for TGFβ induced SMAD signaling, it is critically required for myofibroblast differentiation. In a series of experiments by Sandbo et al, blocking of the ROCK kinase pathway (by Y27632) or blocking actin polymerization (by latrunculin B) both prevented TGFβ-induced expression of fibroblasts αSMA without affecting SMAD-dependent gene expression.[382]

3.2. Anti-fibrotic effects of ROCK in other organ systems

ROCK inhibitors and their anti-fibrotic actions have been studied extensively in experimental **pulmonary fibrosis**. In bleomycin-induced murine models of pulmonary fibrosis, ROCK inhibition has been shown to prevent accumulation of fibrotic tissue and even regress already established fibrosis by interfering with both CTGF and TGFβ signaling, reducing myofibroblast differentiation and by inducing myofibroblast apoptosis.[383-385]

In murine models of **cardiac fibrosis**, ROCK1 haploinsufficient mice developed less perivascular fibrosis in four different models of fibrosis, including angiotensin II infusion, N-nitro-L-argenin methylester treatment, transaortic constriction and fibrosis induced by myocardial infarction. Effects were associated with a reduced CTGF and TGFβ expression.[386]

Fasudil, a non-selective ROCK inhibitor, showed anti-fibrotic properties in a rat model of type 2 diabetes-induced **liver fibrosis** by reducing (myo)fibroblast activation and TGFβ expression.[387] Similarly, Y27632 reduced hepatic fibrosis in a dimethylnitrosamine-induced rat model.[388]

In experimental **renal fibrosis**, both fasudil and Y27632 reduced accumulation of tubulointerstitial fibrosis in a rat model of unilateral urethral obstruction (UUO).[389,390] Fasudil showed additional anti-fibrotic efficacy in a rat model of diabetic nephropathy, with similar efficacy to losartan in this model.[391] Retroperitoneal fibrosis, a life-treatening complication of long standing renal replacement therapy, is also alleviated by ROCK inhibitors. Y27632 showed efficacy in an chlorhexidin-induced rat model of this complication by inhibiting both fibrosis and angiogenesis through reducing TGFβ and VEGF expression.[392]

Finally, ROCK activity also seems to play a role in dermal fibrosis with skin fibroblasts isolated from scleroderma patients showing increased activity. ROCK inhibition prevented TGFβ-induced differentiation and ECM production in these cells.[393]

4. General introduction to rho kinase inhibitors and associated problems

Although several ROCK inhibitors have been developed, only two have been frequently used in *in vitro* and *in vivo* experiments.

Fasudil (HA-1077) is an isoquinolone derivative and targets the ROCK ATP-dependent kinase domain. Similarly, Y27632 is a pyridine derivative that also functions in an ATP-competitive way. Both inhibit ROCK1 and ROCK2 in equimolar concentrations and although they are frequently used in experimental set-ups, several associated problems limit their clinical applicability. [371,394,395]

First of all, both compounds are not very potent ROCK inhibitors with IC50s ranging in the micromolar range, where other clinically approved kinase inhibitors function in the nanomolar range.[395] Secondly, at the concentrations they have to be used both are generally non-selective for ROCK inhibiting other kinases that have similar cellular functions. Y27632, for instance, has been shown to inhibit PKC-related kinase-2, protein kinase N and citron kinase at the concentrations used to study ROCK inhibition, while fasudil inhibits protein kinase A and PKC. [371,396] Additionally, in a model of pulmonary fibrosis, anti-fibrotic properties of Y27632 were associated with reduced phophorylation of SMAD2/3, suggesting non-selective effects on canonical TGFβ signaling.[385]

Overall safety with these systemic ROCK inhibitors is an important concern. Y27632 was removed early from clinical trials because of toxicity. Fasudil, however, has been approved and is on the market in Japan and China since 1995 for treating cerebral vasospasm. Although it appears to be generally well-

tolerated with no increased adverse event rate in a meta-analysis of several trials, concerns about cardiovascular side-effects with more potent ROCK inhibitors remain. Possible side-effects of systemic ROCK inhibition include systemic hypotension and an increased risk of hemorrhage.[371 397]

In conclusion, ROCK signaling forms an attractive target for direct anti-fibrotic therapy in intestinal fibrosis. However, current available inhibitors are not potent enough to achieve maximal effect and have important side-effects.

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CHAPTER II

AIMS AND RESEARCH OBJECTIVES

I. General aims

Prevention and treatment of fibrostenotic complications remain among the biggest therapeutic challenges in current IBD management. The disease mechanics that lead to intestinal fibrosis are incompletely understood and this translates into a lack of available anti-fibrotic agents. Moreover, due to difficulties identifying patients at risk for developing fibrostenotic complications, construction of clinical trials to further explore the few agents that are available has proven next to impossible.

The general aims of this work are:

1. The pre-clinical evaluation of a new target (rho kinases) to prevent and treat intestinal fibrosis in experimental models of IBD
2. Identifying new genetic or biomarkers of intestinal fibrosis in Crohn's disease

II. Specific research questions

The first part of this work is focussed on Rho kinases and their role in intestinal fibrosis (**Chapter III.1**). Rho kinases represent an attractive therapeutic target for this disease complication as they are involved in cellular processes that contribute to fibrogenesis. Moreover, preliminary work in fibrotic complications of other organ systems than the gut has provided evidence for Rho kinase inhibition in the treatment of fibrosis. Systemic side-effects, however, limit their clinical applicability.

The specific research questions for this part are:

- Examine the role of Rho kinases in IBD-related intestinal fibrosis
- Test the applicability and safety of a locally acting Rho kinase inhibitor in mice
- Evaluate the efficacy of this locally acting Rho kinase inhibitor in murine models of intestinal fibrosis
- Identify the cellular mechanisms affected by Rho kinase inhibition and which of them are involved in its anti-fibrotic function
- Evaluate the anti-inflammatory effect of Rho kinase inhibition in murine models of intestinal inflammation
- Examine the efficacy of Rho kinase inhibition in *ex vivo* models of IBD

In a second part of this thesis, focus will shift towards investigating genetic risk factors of fibrostenosing CD which may serve a dual purpose: first to help identify other previously unknown pathways involved in the pathophysiology of intestinal fibrosis that might become attractive targets for future therapies. Secondly, specific genetic factors may help identifying patients at risk for inclusion in clinical trials (**Chapter III.2**).

The specific research questions for this part were

- Identify genetic variants associated with early fibrostenotic disease
- Evaluate the influence of these variants on the time to development of fibrosis
- Examine the expression of associated candidate genes in intestinal fibroblasts *in vitro*

In the third chapter (**Chapter III.3**) of this work, finding a serum biomarker for intestinal fibrosis will be the main focus. Finding such a biomarker is of pivotal importance for the successful design of clinical trials researching anti-fibrotic agents in IBD. It can help identify patients at risk that are preferably included in such trials and might be useful as an intermediate endpoint.

The specific research questions for this part were:

- identification of a serum biomarker for fibrostenotic CD
- evaluate the role of N-glycolysated proteins (glycomics) in intestinal fibrosis

Similarly controversial in the treatment of IBD, is the use of corticosteroids for induction of remission. The initial evidence is based on clinical trials dating back from the 1950s and has never been challenged since. In the last chapter of this thesis (**Chapter III.4**) an answer was sought for this controversy by exploring the rationale for the use of corticosteroids in the induction of remission in UC is explored by performing a systemic review and meta-analysis in accordance with the Cochrane methodology.

The specific research questions for this part were:

- To evaluate the efficacy and safety of oral corticosteroids used for induction of remission in active UC
- Comparing efficacy and safety for inducing remission in UC between
    1. Systemic corticosteroids and locally active agents
    2. High dose and lower dosed corticosteroid regiments
    3. Oral corticosteroids and other active treatments

4. Oral corticosteroids and topical formulations

CHAPTER III

Results

I. Treatment of intestinal fibrosis in experimental inflammatory bowel disease by the pleiotropic actions of a local Rho kinase inhibitor

Running title: Local ROCK inhibition resolves intestinal fibrosis

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Based on:

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ABSTRACT

BACKGROUND: Intestinal fibrosis resulting in (sub)obstruction is a common complication of Crohn's disease (CD). Rho kinases (ROCKs) play multiple roles in TGFβ-induced myofibroblast activation that could be therapeutic targets. Because systemic ROCK inhibition causes cardiovascular side effects, we evaluated the effects of a locally acting ROCK inhibitor (AMA0825) on intestinal fibrosis.

METHODS: Fibrosis was assessed in mouse models using dextran sulfate sodium (DSS) and adoptive T-cell transfer. The in vitro and ex vivo effects of AMA0825 were studied in different cell types and in CD biopsy cultures.

RESULTS: ROCK is expressed in fibroblastic, epithelial, endothelial, and muscle cells of the human intestinal tract and is activated in inflamed and fibrotic tissue. Prophylactic treatment with AMA0825 inhibited myofibroblast accumulation, expression of pro-fibrotic factors, and accumulation of fibrotic tissue without affecting clinical disease activity and histologic inflammation in 2 models of fibrosis. ROCK inhibition reversed established fibrosis in a chronic DSS model and impeded ex vivo pro-fibrotic protein secretion from stenotic CD biopsies. AMA0825 reduced TGFβ1-induced activation of myocardin-related transcription factor (MRTF) and p38 mitogen-activated protein kinase (MAPK), down-regulating matrix metalloproteinases, collagen, and IL6 secretion from fibroblasts. In these cells, ROCK inhibition potentiated autophagy, which was required for the observed reduction in collagen and IL6 production. AMA0825 did not affect pro-inflammatory cytokine secretion from other ROCK-positive cell types, corroborating the selective in vivo effect on fibrosis.

CONCLUSIONS: Local ROCK inhibition prevents and reverses intestinal fibrosis by diminishing MRTF and p38 MAPK activation and increasing autophagy in fibroblasts. Overall, our results show that local ROCK inhibition is promising for counteracting fibrosis as an add-on therapy for CD.

Keywords: colitis, inflammatory bowel disease, Crohn's Disease, fibroblast, autophagy

INTRODUCTION

In patients with ileal Crohn's disease (CD), and to a lesser degree in those suffering from colonic CD or

ulcerative colitis (UC), recurrent episodes of inflammation followed by mucosal healing cause the mucosal and submucosal deposition of extracellular matrix (ECM), which progressively leads to structural fibrosis. Ultimately, up to one third of CD patients develop an end-stage fibrotic dis- ease characterized by intestinal strictures, luminal stenosis, and organ failure.[1] Preventing or reversing ECM deposition in inflammatory bowel disease (IBD) is a major therapeutic challenge. Surgical resection with loss of viable intestinal tissue is required in 80% of cases, with recurrence rates of up to 70%.[2] Although fibrosis is initiated and propagated by recurrent inflammation, suppressing inflammation alone does not halt the progression of this disease, nor does it reverse established fibrosis.[3] Thus, there is an unmet medical need for anti-fibrotic therapies targeting other pathways involved in the pathophysiology of intestinal fibrosis. Although the US Food and Drug Administration recently approved anti-fibrotic agents for idiopathic pulmonary fibrosis, there is no treatment to halt fibrosis in IBD.[4]

One of the processes that could be targeted by anti-fibrotic therapy is the transition of quiescent mucosal fibroblasts into activated myofibroblasts, which are key effector cells of intestinal fibrosis.[5] Both inflammation-induced factors (eg, transforming growth factor-β [TGFβ] and interleukin 6 [IL6]) and mechanical stimuli (matrix stiffness) induce this fibroblast transition. The resultant activated effector cells produce ECM components (eg, collagens), remodeling enzymes such as matrix metal- loproteinases (MMPs), and pro-fibrotic cytokines, including TGFβ1 and IL6. [6] An important hallmark of myofibroblast formation is the conformational change in the cytoskeleton, which is characterized by the appearance of a highly structured scaffold of actin stress fibers and intermediate filaments that enhance cell mobility and facilitate cell activation. Also, epithelial-to-mesenchymal transition (EMT), through which epithelial cells lose their polarized phenotype and transform into myofibroblasts, is an important source of effector cells contributing to intestinal fibrosis. [7]

Anti-fibrotic therapy could also target macro-autophagy in fibroblasts. Autophagy is an evolutionarily conserved mechanism that maintains cellular homeostasis by degrading and recycling proteins and damaged organelles. Poly- morphisms in autophagy-related genes have been associated with IBD, but their role in the pathophysiology of fibrosis remains unclear.[8] Nonetheless, impairment of the autophagic response has been associated with progression of fibrotic diseases in other organs. [9]

The Rho-associated coiled-coil-containing protein kinases (ROCK) are serine/threonine kinases involved in cytoskeletal organization, EMT, and autophagy, and they are therefore potential candidates for anti-fibrotic therapy. [10,11] Two ROCK isoforms have been identified (ROCK1 and 2), both of which are ubiquitously expressed and have a high degree of sequence similarity in human and mouse,

especially in their kinase domains.[12] Various proteins are phosphorylated after ROCK1/2 is activated by RhoA-GTP, such as myosin light chain, which leads to actin polymerization, appearance of stress fibers, and decrease in the cellular concentration of free globular actin. Consequently, myocardin-related transcription factors (MRTFs), sequestered in the cytosol by binding to globular actin, translocate to the nucleus and activate genes involved in cell differentiation and cytoskeletal organization. [12]

Rho kinase inhibition was recently proposed as a treatment for pulmonary fibrosis. However, because of the development of important side effects, such as symptomatic arterial hypotension, its use warrants great caution when these compounds are able to reach the systemic circulation. [13] Therefore, we investigated the effects of the ROCK inhibitor AMA0825, which has a long retention time in the gut but is quickly degraded by esterases in the systemic circulation.

MATERIALS AND METHODS

Part of the materials and methods are provided as a supplementary file.

Ethics statements. The use of patient material was approved by the Ethics Committee of Ghent University Hospital (EC/2015/1145). Written informed consent was obtained from all participants. Mice were housed in the animal facility laboratory at Ghent University Hospital (Ghent, Belgium) according to the institutional animal health care guidelines. This study was approved by the Institutional Review Board of the Faculty of Medicine and Health Sciences of Ghent University (ECD/ 2015/03).

Patient samples. To determine ROCK activity, biopsies were collected during colonoscopy from the ileum of healthy controls and from CD and UC patients with active inflammatory disease. Mucosal tissue from patients with fibrostenotic disease was obtained from ileal resection material from both stenotic regions and non-stenotic regions at the border of the stenosis (see Supplementary Table 1 for patient characteristics). The presence of stenosis was confirmed by the surgeon. The area of stenosis was identified by the pathologist by macroscopic evaluation and was confirmed by histology. Likewise, areas outside the stenosis were identified. CD was diagnosed based on clinical, endoscopic, and histologic criteria. Patients with active inflammatory disease were defined as those with a Crohn's Disease Activity Index >150 and with active inflammation according to the physician performing the endoscopy. Fibrostenotic disease was defined according to the Montreal classification and confirmation of fibrotic stenosis by CT/MRI imaging. For the isolation of fibroblasts, mucosal strips from resected ileal specimens were obtained from CD patients (from both stenotic and non- stenotic

regions as described earlier) and from non-IBD controls undergoing ileocaecal resection for fibrostenotic disease or colon carcinoma, respectively. For ex vivo cultures, 6 ileal stenotic biopsies per patient were obtained during colonoscopy and immediately placed in RPMI1640 medium supplemented with 10% fetal bovine serum, 10,000 U/mL penicillin, 10,000 mg/mL streptomycin, and 200 mg/ mL gentamycin (all from Life Technologies, Ghent, Belgium).

Colitis induction in mice. Seven-week-old male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in open cages in a temperature-controlled room at 20 C with a 12 hour–12 hour dark-light cycle. Animals had free access to water and commercial chow (mouse maintenance chow; Carfil Labofood, Belgium). To induce acute colitis, mice received 2.5% dextran sulfate sodium (DSS) in their drinking water for 7 days, followed by 2 days of normal drinking water. Intestinal fibrosis was induced by administering 2.5% DSS in the drinking water for 1 week, followed by 2 weeks of normal drinking water. This cycle was repeated 3 times.5 During this experiment, groups of mice were sacrificed 6, 9, and 12 weeks after their first exposure to DSS. In a second model of intestinal fibrosis, 8-week-old female CB-17 mice with severe combined immunodeficiency (SCID) were purchased from Charles River Laboratories and housed in individually ventilated cages. Colitis was induced by intraperitoneal (IP) injection of $1x10^6$ CD4+CD25-CD62L+ naive T cells isolated from the spleens of Balb/C mice as previously described.[14] Mice were sacrificed 7 weeks after adoptive transfer.

Prophylactic and therapeutic treatment. Intestinal fibrosis was induced in 7-week-old male C57BL/ 6J mice as described earlier. In the preventive model, mice were randomized and treated daily from the first day of DSS administration by oral gavage with a 20/80 (v/v) mixture of AMA0825 in propylene glycol (Sigma-Aldrich, Overijse, Belgium) and water, or with the vehicle. Mice were sacrificed 9 weeks after the first DSS administration. In the therapeutic model, treatment started after the second cycle of DSS. Mice were sacrificed 6, 9, or 12 weeks after the first DSS cycle. In the adoptive T-cell transfer model, colitis was induced as described earlier.[14] Clinically identifiable colitis, defined as >10% drop in body weight and diarrhea, began developing from week 2. From that time, mice were randomized and treated with AMA0825 by oral gavage in combination with IP administration of anti-tumor necrosis factor (TNF) IgG1 (clone TN3-19.12, BioXcell, West Lebanon, NH) or an isotype control (BioXcell). Mice were sacrificed 45 days after the induction of colitis. In all experiments, the distal, mid, and proximal

sections of the colon were obtained for histologic analysis, RNA extraction, and cytokine measurements.

Biopsy cultures. Biopsies were washed 3 times and cultured in RPMI1640 medium supplemented with 1% fetal bovine serum, 10,000 U/ mL penicillin, 10,000 mg/mL streptomycin, and 50 mg/mL gentamycin together with either AMA0825 or an equal volume of dimethyl sulfoxide. After 24 hours of incubation, the supernatant was collected, cleared by centrifugation and stored at –80 C. Each treatment was performed in triplicate (1 biopsy per well).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad, San Diego, CA) or SPSS Statistics version 22 (Chicago, IL). Temporal changes in body weight were compared between groups using a linear mixed model. Student's t test was used to compare differences between 2 groups for normally distributed data. Data that were not normally distributed were log-transformed or analyzed by Mann–Whitney U test. Paired biopsy culture data were compared using mixed models. Two-tailed probabilities were calculated and P values <.05 were considered significant.

RESULTS

ROCK is activated in inflamed and fibrotic tissue

Based on the hypothesis that targeting ROCK could be useful for treating intestinal fibrosis, its kinase activity was measured in representative tissues. Ileal biopsies were obtained from healthy controls, CD patients with inflammatory disease, and UC patients with backwash ileitis. ROCK activity was 14-fold higher in biopsy lysates of inflamed samples compared with healthy tissue and backwash ileitis (Figure 1A, left panel). ROCK activity was higher in mucosal lysates of stenotic tissue (devoid of signs of active inflammation on histopathology), than in non-stenotic tissue adjacent to the stenosis in the same patient (Figure 1A, right panel). Epithelial cells, fibroblasts, endothelial cells, and muscle cells were immunopositive for ROCK (Figure 1B).

Next we determined whether the commonly used animal models of intestinal fibrosis are representative of the ROCK activity associated with inflammation and fibrosis observed in CD samples. Oral administration of DSS to C57BL/6J mice over 7 days led to acute inflammatory colitis with minor histologic signs of ECM deposition (Figure 1C-E) accompanied by high ROCK activity in the colon (Figure 1F). With repeated cycles of DSS, mice gradually developed chronic signs of colitis and increased ECM deposition (Figure 1C-E). ROCK activity diminished as the colitis became chronic but remained elevated 2 and 5 weeks after the last adminis-tration of DSS (Figure 1F). Seven weeks after adoptive T-cell transfer, ROCK activity was also increased in fibrotic colonic tissue from SCID mice (Figure 1C-F). Although these mouse models mimic colonic fibrosis without ileal pathology, these data indicate that the models are suitable for studying fibrosis and ROCK inhibition in vivo.

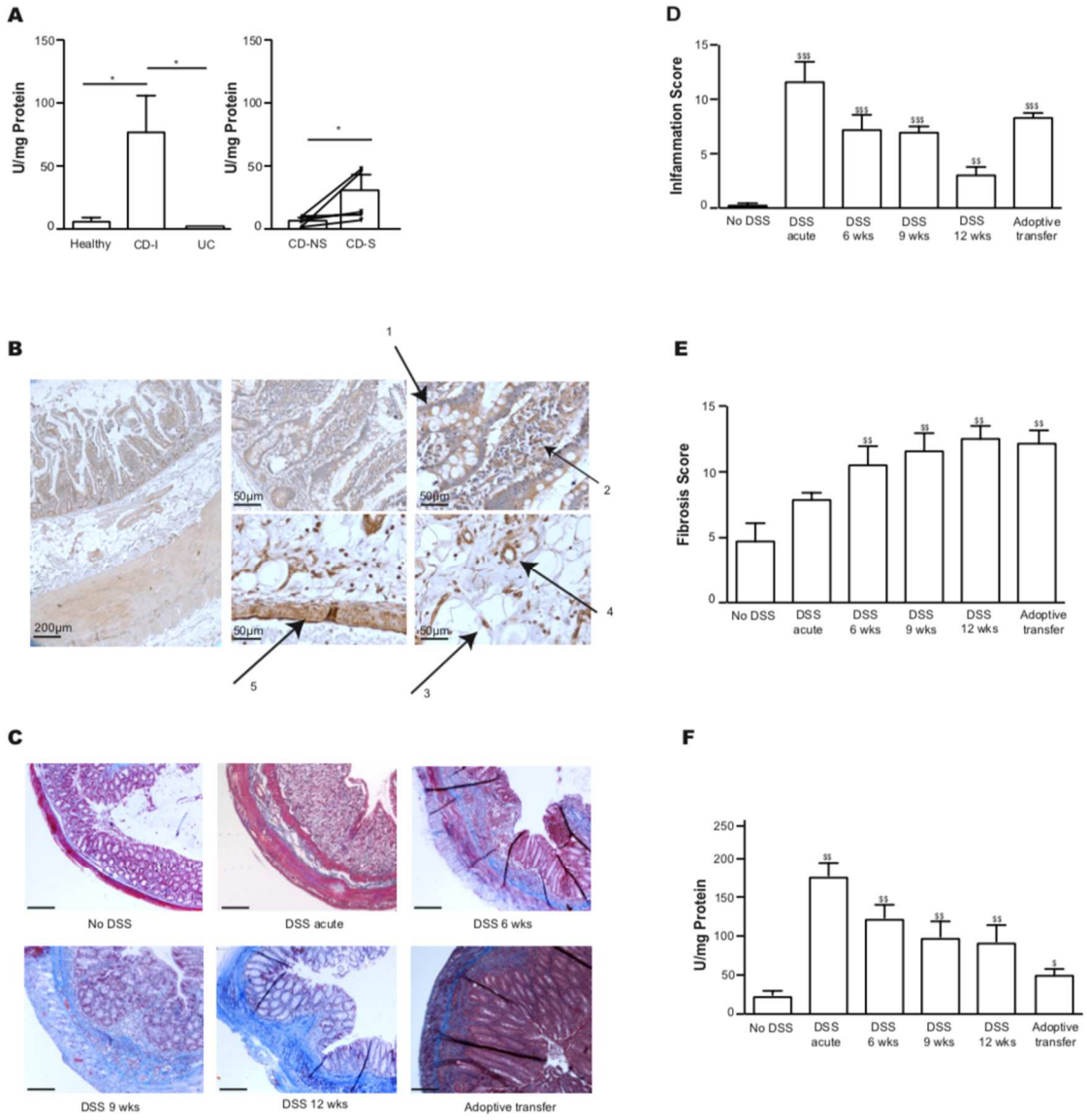

Figure 1. ROCK activity is elevated in inflamed and fibrotic intestinal tissue. ROCK activity is elevated in inflamed and fibrotic intestinal tissue. (A) ROCK activity was measured by immunoassay in biopsies of healthy ileum (n=4), inflamed ileum of CD patients (CD-I, n=5), ileum from UC patients with backwash ileitis (UC, n=3), and the mucosa of the resected ileum of CD patients with fibrostenotic disease, collected from non-stenotic (CD-NS, n=5) and stenotic regions (CD-S, n=5). (B) Immunohistochemical staining for ROCK in the stenotic ileum of a CD patient showing immunopositivity in (1) epithelial cells, (2) subepithelial fibroblasts, (3) submucosal fibroblasts, (4) endothelial cells, and (5) muscle cells. (C) Representative Masson's trichrome images of the distal colon in different stages of the development of chronic colitis induced by DSS 6, 9, and 12 weeks (wks) after the first DSS administration and after adoptive T cell transfer (n=8 in each group, bar=200 µm). (D) Colonic inflammation and (E) fibrosis scores. (F) ROCK activity in full- thickness distal colonic tissue during different stages

of chronic DSS and adoptive T cell transfer. Data are the mean ± SEM. *P < .05; $P < .05, $$P < .01, $$$P < .001 compared with the group not treated with DSS.

Prophylactic administration of AMA0825 prevents the accumulation of fibrotic tissue in experimental colitis

AMA0825 is a highly selective, small-molecule inhibitor of ROCK 1 and ROCK 2 (IC50<50 pM). Upon reaching the systemic circulation, AMA0825 is rapidly cleaved by para-oxonases, reducing its half-life to <20 minutes. Consequently, though oral administration of 3 mg/kg AMA0825 in mice led to colonic drug levels above the functionally active concentrations, it did not cause cardiovascular side effects or influence ROCK activity in other organs (Supplementary Figure 1).

Having established the potency, selectivity, and local action profile of AMA0825, we studied whether oral administration of AMA0825 prevents the rise in colonic ROCK activity upon DSS challenge. Prophylactic treatment with 3 mg/kg per day resulted in a 2-fold decrease in ROCK activity in the distal colon (96.3 ± 19.8 U/mg protein in the placebo group vs 49.1 ± 8.7 U/mg protein in treated mice; P < .05). However, these results should be interpreted with caution because residual AMA0825 in the tissue lysates could have interfered with the activity assay. AMA0825- treated mice developed less intestinal fibrosis, as shown by 4 parameters: (1) reduction in the colonic weight/length ratio (Figure 2A); (2) deposition of ECM observed by histology (Figure 2B); (3) tissue expression of collagen I (Col1a1) and connective tissue growth factor (Ctgf); and (4) production of TGFβ1 and IL6 in the distal colon (Figure 2C). To characterize the MMP expression profile induced by DSS, we used the Luminex (Bio-Rad Laboratories, Temse, Belgium) panel of MMPs. Fibrosis elicited by DSS was accompanied by increased expression of MMP-2, -3, -8, -9, and -12, whereas MMP-7 and -13 remained at baseline levels. AMA0825 treatment inhibited the expression of MMP-2, -3, and -9, while production of MMP-8 and -12 was unaffected (Figure 2D). In addition, AMA0825-treated mice exhibited reduced αSMA immunopositivity in the mucosa and submucosa compared with placebo-treated mice, indi- cating a decrease in the number of intestinal myofibroblasts (Figure 2E).

The attenuation of acute inflammatory responses caused by repeated DSS administration could account for the observed changes in fibrosis. Surprisingly, prevention of fibrosis was accompanied by a reduction in colonic IL6 levels (Figure 2C) but not by amelioration of body weight loss, colonic myeloperoxidase activity, histologically observable inflammation, expression of pro-inflammatory markers, serum IL6 levels, or macrophage infiltration (Supplementary Figure 2). In acute models of colitis elicited by DSS or TNBS (2, 4, 6-trinitrobenzenesulfonic acid), body weight loss was ameliorated by AMA0825 treatment but there was no improvement of inflammation as seen in his-tology, colon

length, or myeloperoxidase activity (Supplementary Figures 3 and 4, respectively). Additionally, during lipopolysaccharide (LPS)-induced small intestinal inflammation and barrier dysfunction, AMA0825 did not significantly affect local or systemic inflammation or barrier leakage (Supplementary Figure 5).[15]

Finally, the effects of 3 mg/kg AMA0825 per day were evaluated in the adoptive T-cell transfer model and were compared and combined with the standard IBD treatment (anti-TNF). AMA0825 alone did not improve body weight, histologically observed inflammation, or production of the proinflammatory cytokines IFNγ, MCP1 and IL1β (Figure 3A-C). However, fibrosis and production of TGFβ and IL6 were significantly reduced, both in AMA0825 monotherapy and when it was combined with anti-TNF (Figure 3D-E). Anti-TNF monotherapy was not associated with a significant reduction of histologically evident intestinal fibrosis, despite improvement in weight evolution, decreased histologic inflammation, and attenuated colonic production of IFNγ, MCP1, IL1β, and IL6. Interestingly, combining anti-TNF with AMA0825 prevented histopathologically observable fibrosis, illustrating the benefit of adding an anti-fibrotic agent to anti-inflammatory therapy.

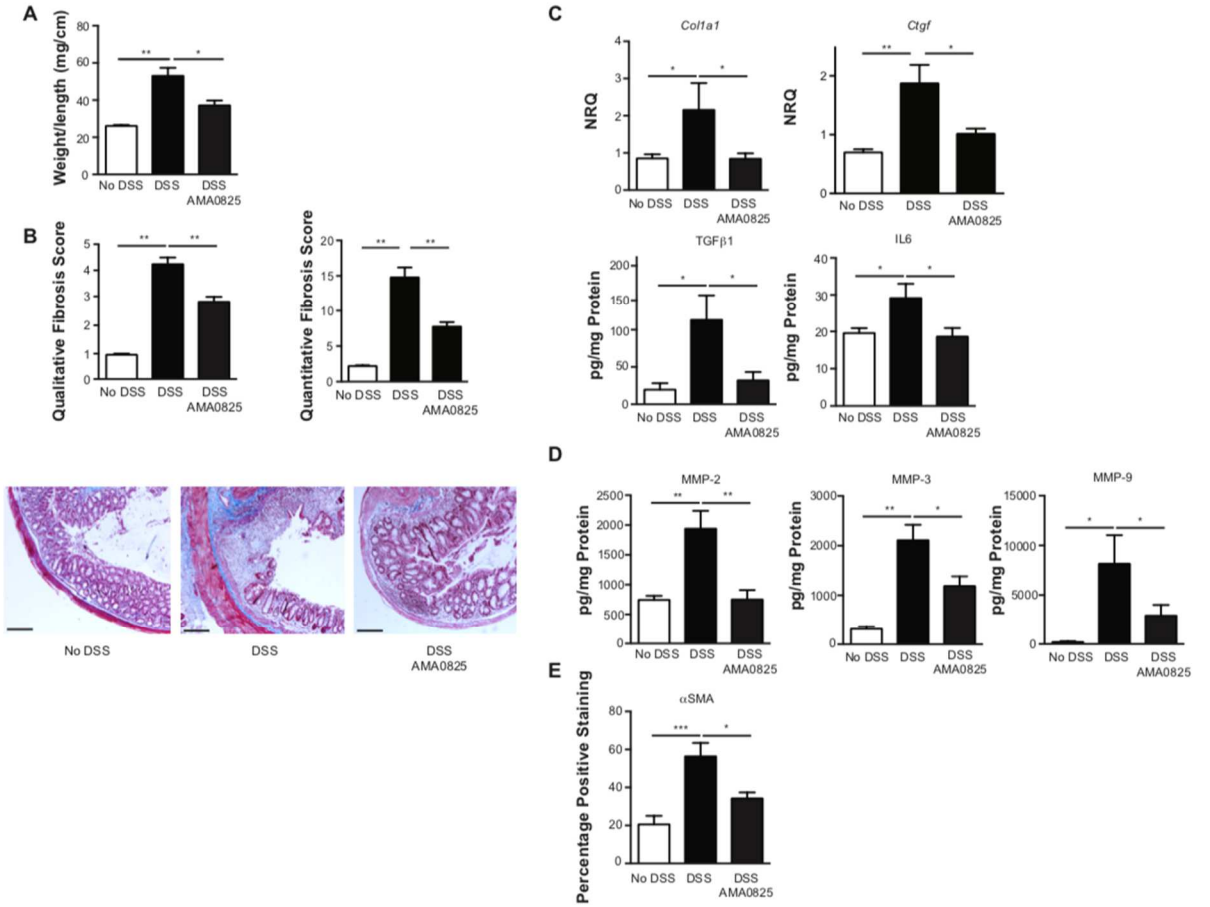

Figure 2. Local ROCK inhibition attenuates the development of fibrosis during chronic DSS-induced colitis. C57BL/6J mice were subjected to 3 cycles of 2.5% DSS for 1 week followed by 2 weeks of recuperation. Mice were treated preventively by oral gavage with 3 mg/kg per day AMA0825 from the start of DSS administration (n=8 in each group). (A) Colonic weight/length ratio. (B) Qualitative and quantitative fibrosis scores, and representative Masson's trichrome images of the distal colon (bar=200 µm). (C) Colonic transcript levels of collagen I (Col1a1) and Ctgf measured by quantitative real-time polymerase chain reaction (qRT-PCR), and colonic protein concentrations of TGFβ1 and IL6. (D) MMP-2, -3 and -9 measured by Luminex bead technology. (E) Quantification of alpha smooth muscle actin (αSMA) by immunohistochemical staining of the distal colon (200x). Data are the mean ± SEM. NRQ, normalized relative quantities. *P < .05, **P < .01, ***P < .001.

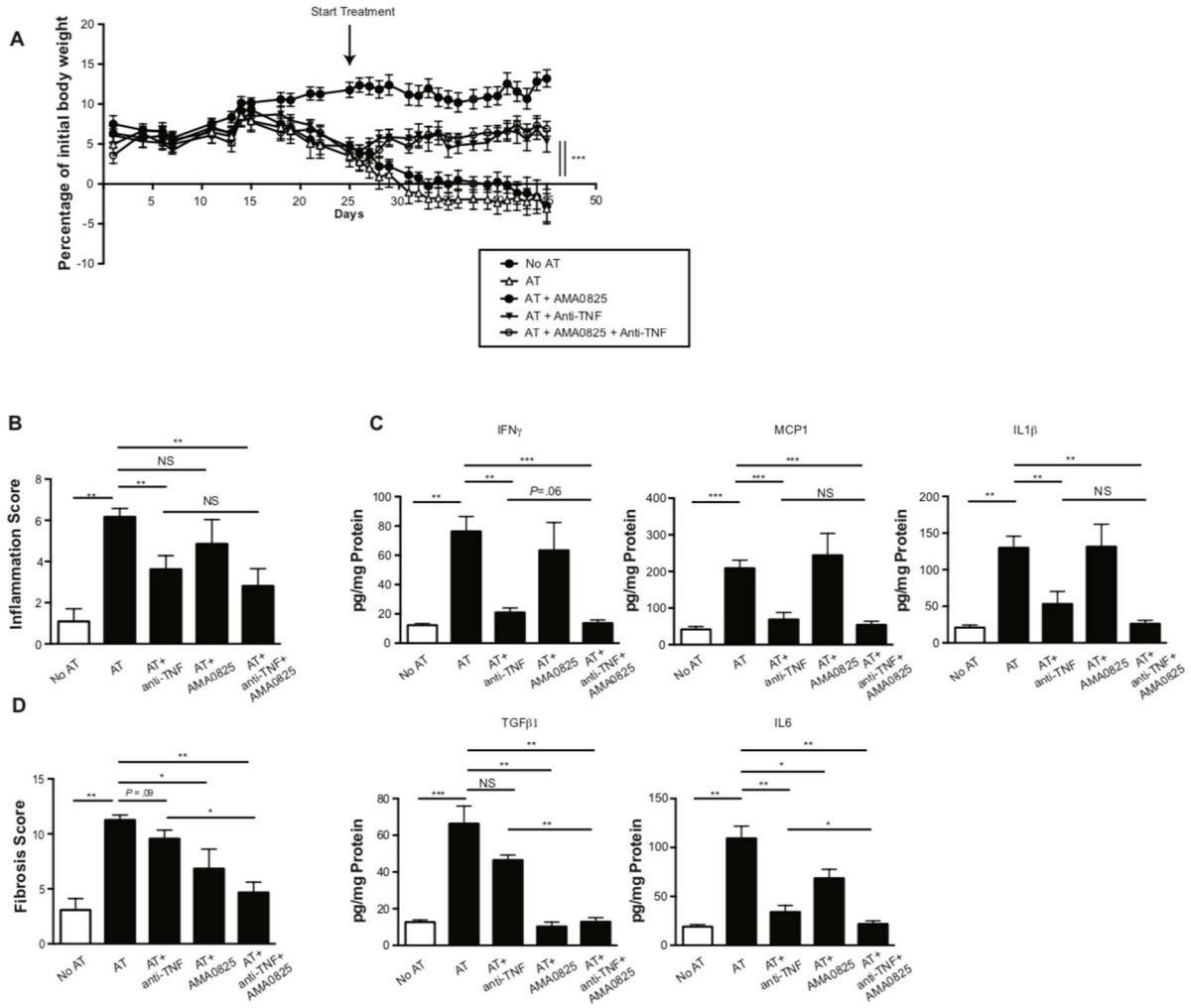

Figure 3. Anti-TNF combined with AMA0825 prevents fibrosis, but anti-TNF alone does not. CD4+CD25-CD62L+ naive T cells were injected IP in CB-17 SCID mice on day 0 (adoptive transfer, AT). Mice

developed symptoms of colitis starting on week 2, at which time therapy was initiated. Mice were treated with 25 mg/kg anti-TNF IP 3 times per week, with 3 mg/kg per day AMA0825 by oral gavage, or with a combination of both (n=10 in each group). Placebo-treated mice received 25 mg/kg IgG1 IP 3 times per week and oral gavage of vehicle. (A) Changes in body weight, (B) histologic inflammation, (C) colonic cytokine concentration, (D) Masson's trichrome-based fibrosis, and (E) colonic TGFβ1 and IL6 concentrations. Data are the mean ± SEM. NS, not significant; *P < .05; **P < .01; ***P < .001.

Local ROCK inhibition by AMA0825 reverses end-stage fibrosis

Because many CD patients have strictures at the time of diagnosis, it is important to know whether local ROCK inhibition can reverse fibrosis. We examined the effect of AMA0825 on mice with established fibrosis using 2 experimental settings (Figure 4A). In both settings, treatment was initiated after 2 cycles of DSS, when substantial fibrotic tissue was already present (Figure 4B). Treatment was continued for 3 or 6 weeks. Because reversing established fibrosis might be more difficult than treating it, we included a group treated with a higher dose. At 10 mg/kg per day, 6 weeks of treatment with AMA0825 significantly reduced the amount of fibrotic tissue (Figure 4B). Colonic levels of IL6, TGFβ1, and MMP-2, -8, -9, and -12 were significantly reduced after 3 and 6 weeks of treatment with 10 mg/kg per day of AMA0825, but MMP-3 expression was unaffected (Figure 4C and D; Supplementary Table 3). These anti-fibrotic changes were associated with reduced αSMA density (Figure 4E).

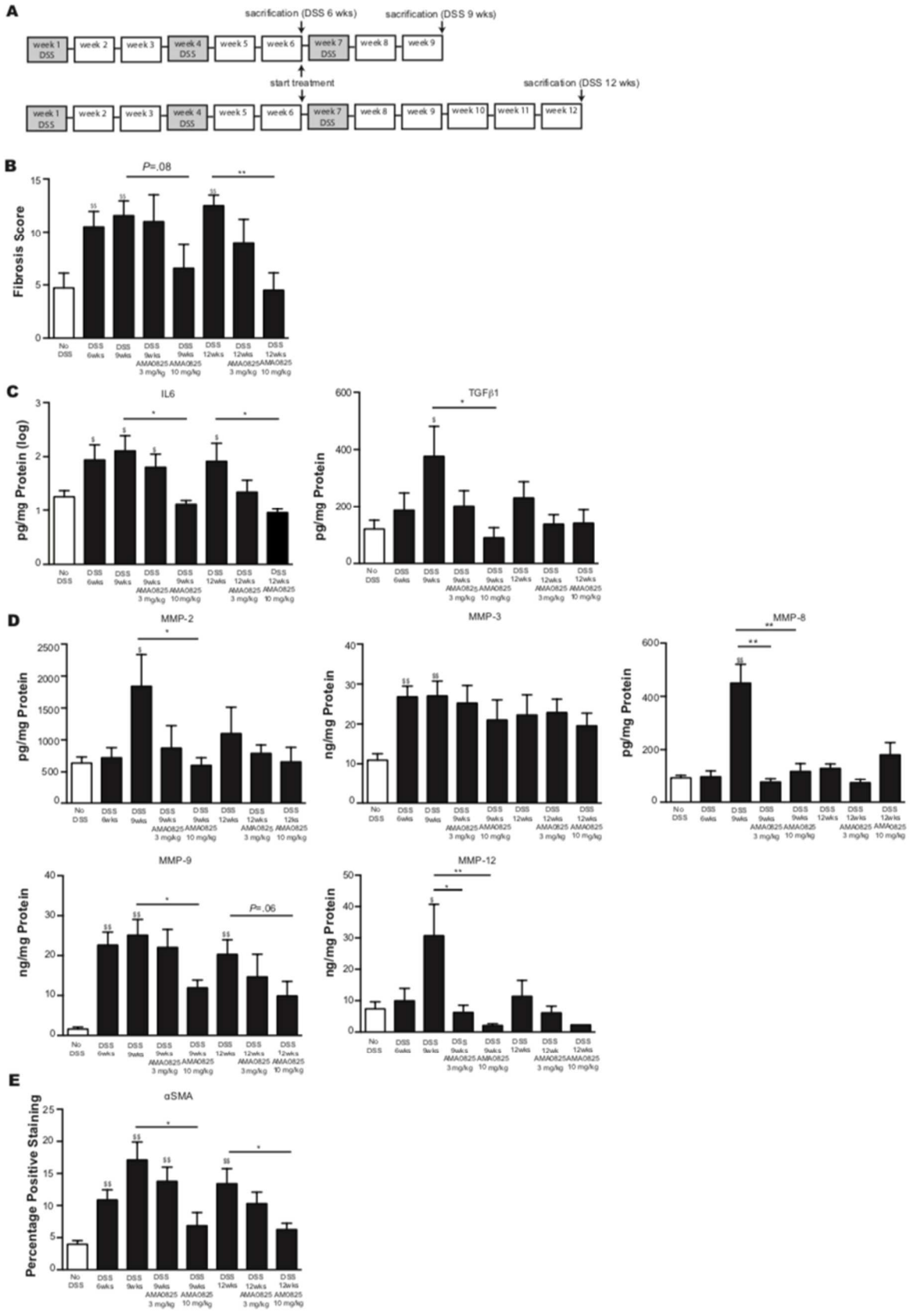

Figure 4. Local inhibition of ROCK by AMA0825 reverses end-stage fibrosis. C57BL/6J mice were subjected to 3 cycles of 2.5% DSS for 1 week followed by 2 weeks of recuperation. (A) Treatment was initiated

on week 6, after completion of 2 cycles of DSS treatment, when intestinal fibrosis was already present. Treatment consisted of 3 or 10 mg/kg of AMA0825 per day, or a placebo, for 3 or 6 weeks (n=7 in each group). (B) Colonic fibrosis assessed by Masson's trichrome staining. (C) Distal colonic protein concentrations of IL6, TGFβ1 and (D) MMP-2, - 3, -8, -9, and -12 measured using Luminex bead technology. (F) Quantification of alpha smooth muscle actin (αSMA) by immunohistochemical staining of the distal colon. Data are the mean ± SEM.*P < .05, **P < .01; $P < .05, $$P < .01 compared with the group not treated with DSS.

ROCK Inhibition Prevents the Activation of Mesenchymal Cells

Because AMA0825 mainly targeted the accumulation of myofibroblasts and the expression of mediators released by activated myofibroblasts in vivo (TGFβ1 and IL6), we first investigated the effects of ROCK inhibition in cultured primary human intestinal fibroblasts (HIF). TGFβ1 induced ROCK activity and IL6 release, which was both dose- dependently inhibited by AMA0825 with an IC50 of 0.1 µmol/L for IL6 release (Figure 5A). Based on lactate dehydrogenase release (data not shown), up to 100 mmol/L AMA0825 caused no toxicity. Proliferation of myofibroblasts, measured by the MTT assay (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), was dose-dependently reduced by AMA0825 at concentrations of 1 µmol/L and higher (data not shown). In addition, ROCK inhibition prevented the TGFβ1-induced transition from fibroblasts to myofibroblasts, quantified by the formation of F-actin- and vimentin-positive stress fibers (Figure 5B). Inhibition of the transition was accompanied by reduced expression of *COL1A1, TGFβ1*, and *CTGF* (Figure 5C) and reduced production of CTGF protein (Figure 5D). In HIFs, TGFβ1 induced the secretion of MMP-2, -3, and -12, while the production of MMP-7, -8, -9, and -13 was unde tected (Figure 5E, Supplementary Table 3). AMA0825 reduced the TGFβ1-induced secretion of MMP-2 and -12, while MMP-3 secretion remained unchanged. Adding AMA0825 48 hours after TGFβ1 stimulation reduced IL6 secretion, indicating the reversibility of the myofibroblast phenotype in HIFs (Figure 5F).

In intestinal smooth muscle cells, TGFβ1 stimulation induced ROCK activity. This induction was dose-dependently reduced by AMA0825, resulting in a significant reduction of IL6 secretion (Supplementary Figure 6). Also, EMT induction in HT29 epithelial cells, which led to the loss of E-cadherin, was significantly inhibited (Supplementary Figure 7).

Finally, we evaluated the dose-range effects of AMA0825 on TNFα-stimulated epithelial cells. At 100 µmol/L, AMA0825 was toxic, but at lower doses up to 10 µmol/L, it suppressed ROCK activity without interfering with IL1β, IL8 or IL6 secretion, nor did it affect the epithelial barrier dysfunction of TNFα-stimulated Caco-2 monolayers (Supplementary Figure 8). Although these findings are in line with the absence of profound anti-inflammatory effects in vivo, previous reports using Y27632, a nonselective

ROCK inhibitor, did show barrier-protective and anti- inflammatory effects.[16] Y27632 was less potent in sup-pressing ROCK activity in epithelial cells (Supplementary Figure 8), and it suppressed IL6 and IL1β secretion only at the highest concentration (100 µmol/L).

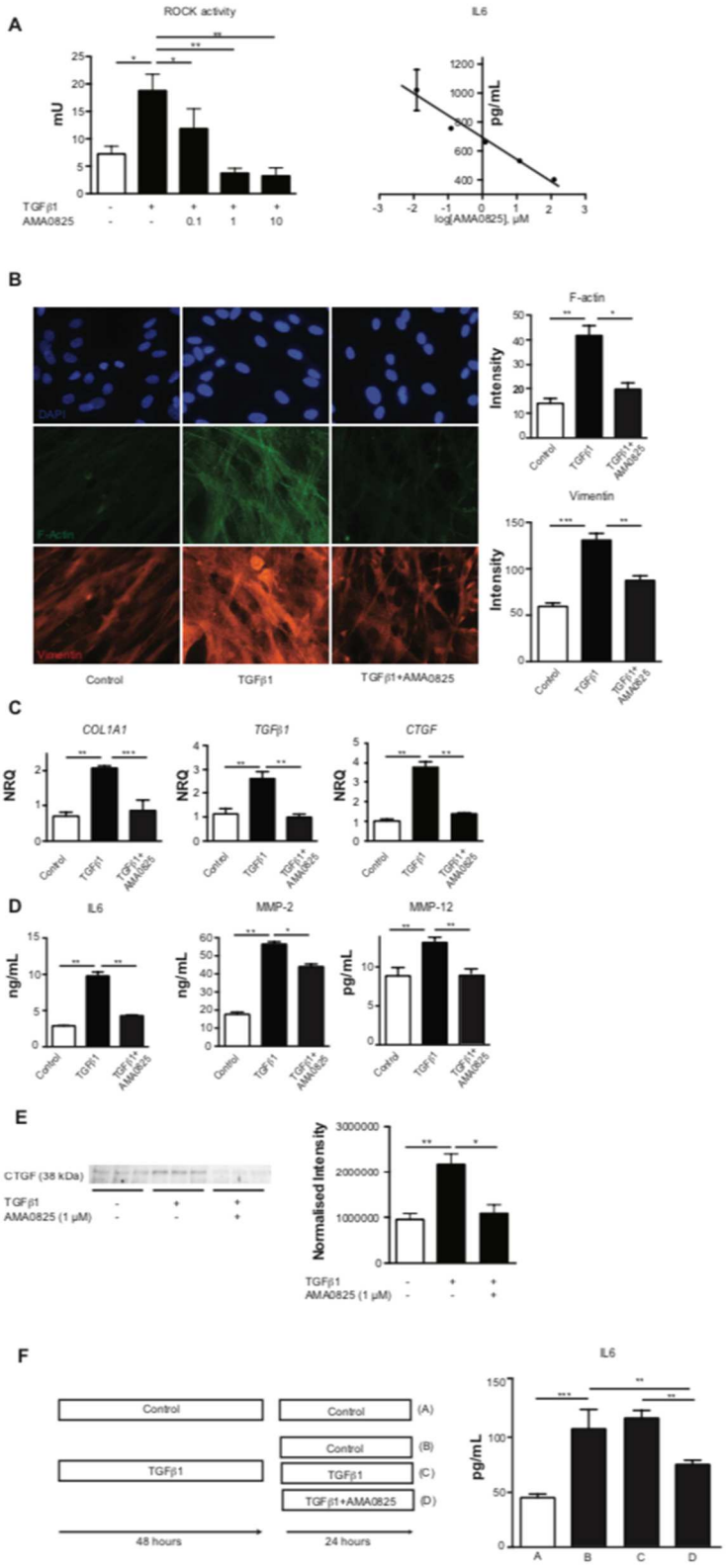

Figure 5. ROCK inhibition abrogates the fibroblast to myofibroblast transition and their subsequent activation. Primary HIFs were stimulated for 48 hours with 1 ng/mL TGFβ1 and a dose-range of AMA0825 and (A) ROCK activity (left panel) and IL6 release (right panel) was measured (n=3 per condition). Next, cells were stimulated with 1 ng/mL TGFβ1, a combination of 1 ng/ mL TGFβ1 and 1 µmol/L AMA0825, or the vehicle control (n=3 per condition). (B) Representative images and quantification of F-actin and vimentin staining. (C) Transcript levels of *COL1A1, TGFβ1*, and *CTGF* analyzed by qRT-PCR. (D) Representative western blot images of CTGF expression. (E) MMP-2 and MMP-12 protein concentrations in supernatant samples measured using Luminex bead technology. (F) The former experiment was repeated using an experimental set-up (left panel) to determine the reversibility of myofibroblast formation by exposing fibro- blasts to 1 ng/mL TGFβ1 or vehicle for 48 hours, after which the supernatant was discarded and the cells were incubated further for 24 hours with vehicle, TGFβ1 alone, or TGFβ1 combined with 1 µmol/ L AMA0825. IL6 protein concentrations (right panel) in supernatant measured using Luminex bead technology. Data are the mean ± SEM. NRQ, normalized relative quantities. *P < .05, **P < .01, ***P < .001.

ROCK Inhibition interferes with MRTF-dependent transcriptional regulation of pro-fibrotic genes and p38 activation

To study how ROCK inhibition decreased the activation of HIFs, we first identified the genes induced by MRTF, a transcription factor that induces Rho-dependent myogenic gene expression in TGFβ1-stimulated cells. TGFβ1 induced the transcription of *COL1A1, ACTA2, TGFβ1*, and *MMP-2*. This induction could be blocked by inhibiting MRTF subtype A with CCG-1423, but IL6 transcription and secretion were not prevented (Supplementary Figure 9A). Next, we characterized the IL6 secretory response of TGFβ1 using selective inhibitors of MEK1 (PD0325901), p38 MAPK (SB203580), JNK (SP600125), and SMAD2/3 (SB431542), all of which are downstream signalling pathways of TGFβ1 signalling. TGFβ1-induced IL6 secretion was counteracted by p38 MAPK and SMAD2/3 inhibition, but not by JNK inhibition, while MEK1 inhibition sensitized the cells for TGFβ1-induced IL6 secretion (Supplementary Figure 9B). Western blot analysis confirmed that TGFβ1 stimulation led to induction of MRTF and to phosphorylation of p38 MAPK and SMAD2/3 (Supplementary Figure 9C-E). Inhibition of ROCK by AMA0825 prevented the induction of p38 phosphorylation and MRTF expression, but not TGFβ1-induced phosphorylation of SMAD2/3.

Autophagy is Induced by ROCK Inhibition

Given the role of autophagy impairment in the development of fibrosis in other organs, we quantified autophagy in intestinal myofibroblasts from AMA0825-treated and untreated mice used in the chronic DSS experiment. Intestinal fibroblasts from AMA0825-treated mice exhibited increased autophagy compared with those isolated from placebo-treated animals (Figure 6A), suggesting that ROCK inhibition amplifies autophagy in fibroblasts in vivo. To confirm these observations in vitro, we examined cultured HIF cells by transmission electron microscopy. We found that TGFβ1 stimulation increased the number of autophagosomes and that co-incubation with AMA0825 increased it further (Figure 6B). Moreover, p62 levels decreased significantly upon AMA0825 stimulation (Figure 6C). Adding the autophagy inhibitor bafilomycin A1, a H+ ATPase inhibitor that inhibits the fusion of autophagosomes with lysosomes, prevented the drop in p62 levels, confirming that AMA0825 specifically induces autophagy in TGFβ1-stimulated fibroblasts (Figure 6C).

Induction of autophagy in myofibroblasts is required for the anti-fibrotic actions of ROCK inhibition

To evaluate the functional relevance of autophagy in the response of TGFβ1-stimulated HIFs to ROCK inhibition, production of collagen and IL6 was assessed in the presence of bafilomycin. Addition of bafilomycin counteracted the reduction of collagen I and IL6 protein production caused by AMA0825 in TGFβ1-stimulated HIFs (Figure 6D). At the transcriptional level, inhibiting autophagy did not affect the inhibition of *COL1A1* expression by AMA0825 (Figure 6E), indicating that the autophagic response induced by AMA0825 leads to post-translational degradation of collagen fibers. Bafilomycin also attenuated the effects of AMA0825 on *IL6* gene transcription, which suggests that an upstream transcription factor controlling *IL6* transcription is targeted for autophagic degradation (Figure 6E). Because phosphorylation of p38 MAPK is responsible for IL6 secretion in HIFs, we examined whether this kinase may also be controlled by autophagy. Indeed, bafilomycin counteracted the inhibition of p38 MAPK phosphorylation by AMA0825 (Supplementary Figure 9D).

To confirm the functional relevance of the autophagy induced by AMA0825, we isolated intestinal fibroblasts from the colon of Atg16l1-deficient mice. These mice carry a hypomorphic (HM) mutation in the CD-associated risk gene Atg16l1 that impairs the normal autophagic response.[17,18] In contrast to the effect of ROCK inhibition on fibroblasts isolated from wild-type mice, it had no effect on TGFβ1-induced IL6 production in Atg16l1HM/HM fibroblasts. This finding validates the importance of the increased autophagic flux in the mechanism of action of AMA0825 (Figure 6F).

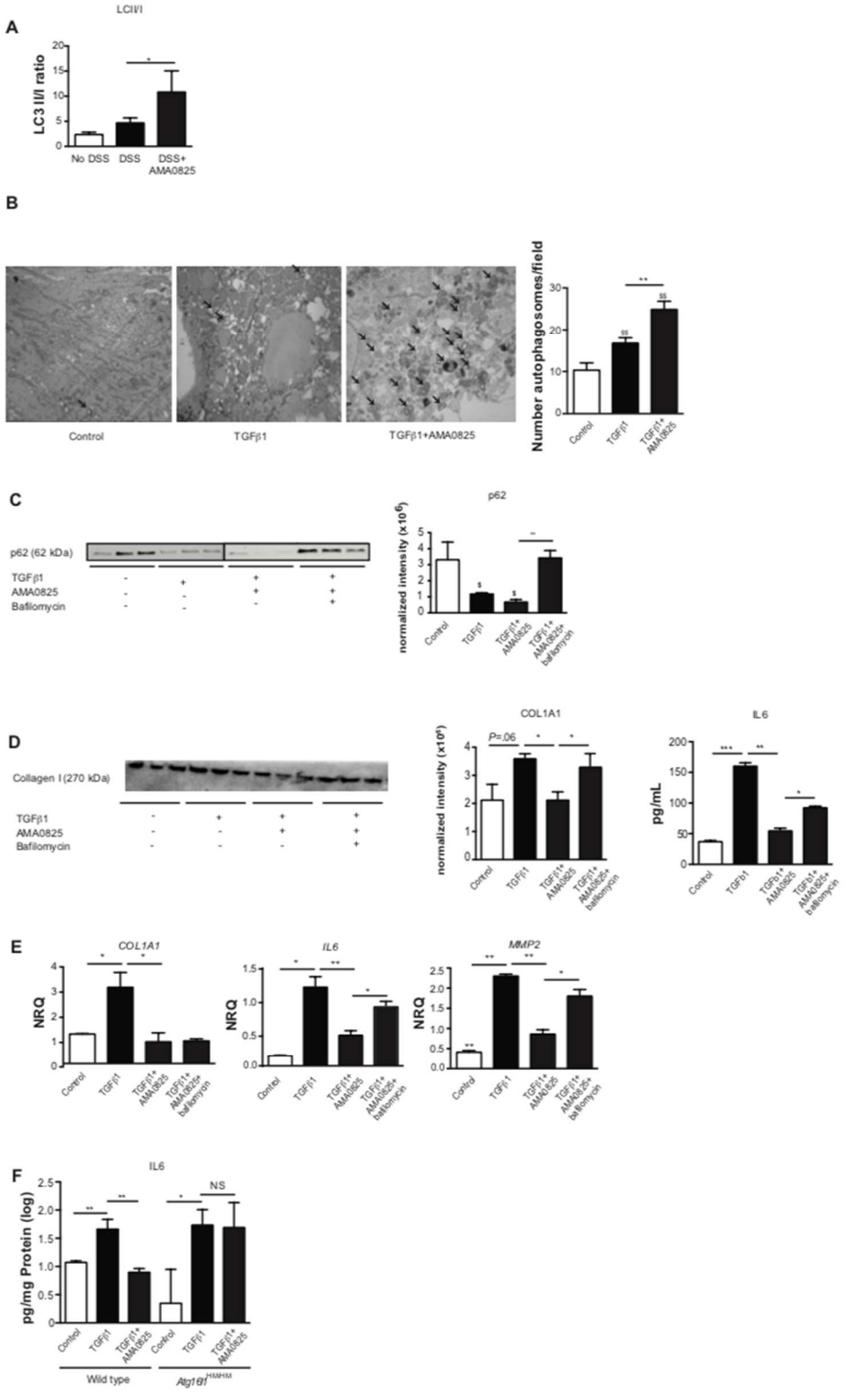

Figure 6. ROCK inhibition by AMA0825 amplifies the autophagic response in fibroblasts and controls collagen, IL6, and MMP expression. (A) Autophagic flux (LC3B-II/I ratio quantified by western blot analysis) in fibroblasts isolated from C57/BL6J mice undergoing repeated cycles of DSS treatment with or without oral treatment with 3 mg/kg per day AMA0825 (n=6 per group). (B) Representative electron microscopy images

(5000x) of primary HIFs treated for 48 hours with 1 ng/mL TGFβ1, a combination of 1 ng/mL TGFβ1 and 1 µmol/L AMA825 or the vehicle control. Autophagosomes (arrows) were counted in 10 cells per condition in 4 images per cell. (C) Primary HIFs were treated for 48 hours with 1 ng/mL TGFβ1 or a combination of TGFβ1 and 1 µmol/L AMA0825 in the presence or absence of 10 nmol/L bafilomycin A1 (n=3 per condition). The expression of p62 and collagen I was quantified by western blot analysis. (D) Collagen I and IL6 secretion was measured using western blot and Luminex bead technology, respectively. (E) Transcript levels of *COL1A1*, *IL6*, and *MMP2* analyzed by qRT-PCR. (F) Protein concentration of IL6 in the supernatant mouse intestinal fibroblasts isolated from Atg16l1 hypomorphic (ATG16L1HM/HM) and wild-type mice (n=4 per group) treated for 48 hours with 1 ng/mL TGFβ1, a combination of 1 ng/mL TGFβ1 and 1 µmol/L AMA0825, or the vehicle control. Data are the mean ± SEM. NRQ, normalized relative quantities; NS, not significant. *P < .05, **P < .01; $P < .05, $$P < .01 compared with the control group.

AMA0825 reduces the secretion of pro-fibrotic mediators from stenotic tissue of CD Patients Ex Vivo

The mucosal tissue overlying fibrotic strictures is biologically active and secretes pro-fibrotic proteins thought to enhance the progression of disease-related strictures.[19] Ex vivo, biopsies collected from the stenotic ileum of CD patients secreted TGFβ1, IL6, and MMP-2, -3, -9, -12, and -13, but MMP-7 and -8 were undetected. Incubating these tissues for 24 hours with AMA0825 significantly diminished the secretion of MMP-2, -3, -12, TGFβ1, and IL6 (Figure 7A), while MMP-9 and -13 levels remained unchanged. The release of IL6 in response to TGFβ1 was 1.5-fold higher in fibroblasts isolated from fibrotic segments of CD patients than in fibroblasts isolated from non-fibrotic segments or from the healthy ileum (Figure 7B), indicating that fibroblasts in stenotic regions are more responsive to TGFβ1. Incubation of the fibroblasts with AMA0825 abolished this sensitizing effect and completely inhibited IL6 release. Together, these data indicate that AMA0825 can halt the function of activated fibroblasts in the stenotic bowels of CD patients.

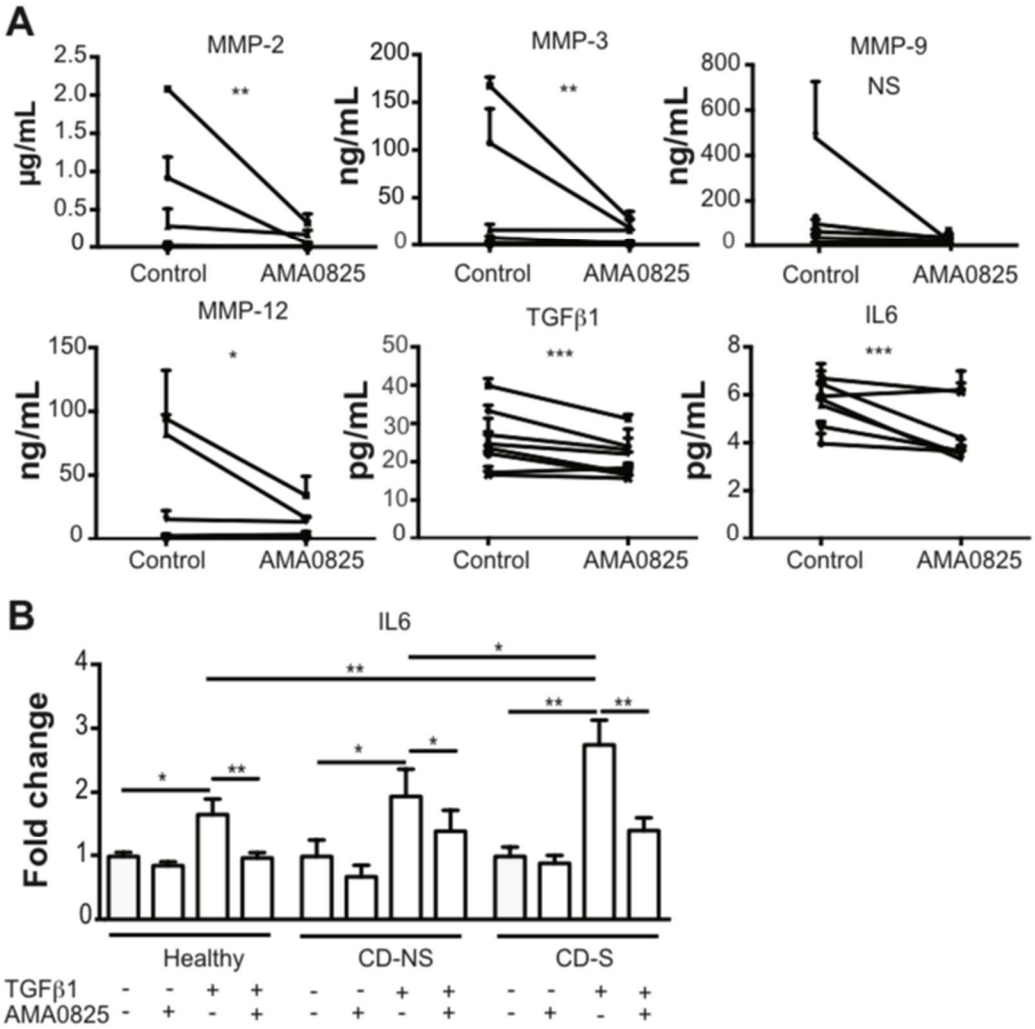

Figure 7. AMA0825 reduces pro-fibrotic protein secretion from human ileal stenotic biopsies and from primary ileal fibroblasts. (A) Ileal biopsies obtained from stenotic regions of CD patients during colonoscopy (n=8) were incubated for 24 hours with 5 µmol/L AMA0825 or vehicle. Levels of MMP-2, -3,-9, -12, TGFβ, and IL6 were determined in the supernatant using Luminex bead technology (n=3 per condition). (B) Fibroblasts were isolated from resection specimens of healthy ileum (n=2) and from non-stenotic (CD-NS) and stenotic (CD-S) resection specimens from CD patients (n=6). Cells were stimulated for 24 hours with 1 ng/mL TGFβ1 in the presence of 1 µmol/ L AMA0825 or the vehicle. The protein concentration of IL6 was determined in the supernatant using Luminex bead technology. Data are the mean ± SEM. NS, not significant. *P < .05, **P < .01, ***P < .001.

DISCUSSION

We describe a pre-clinical study demonstrating that a locally acting ROCK inhibitor prevents and reverses intestinal fibrosis by targeting different cellular mechanisms, particularly by diminishing TGFβ1-induced MRTF and p38 MAPK activation and increasing autophagy in fibroblasts.

Intestinal fibrosis causing bowel strictures and stenosis is associated with significant morbidity and mortality in patients with IBD. Despite recent advances in anti-inflammatory therapy, the incidence of complications in IBD has not declined, suggesting that control of recurrent inflammation does not limit the progression of fibrosis.[20] On the other hand, given that a substantial proportion of CD patients display some intestinal fibrosis at the time of diagnosis, compounds that reverse established fibrosis would be of great value too.[20] In this paper, we provide evidence that ROCK is a promising target for anti-fibrotic therapy. This kinase is necessary for the TGFβ1-induced transition of fibroblasts to activated myofibroblasts by playing a role in controlling myogenic differentiation through MRTF-, p38 MAPK- and autophagy-dependent mechanisms.

First, we demonstrated that ROCK is expressed in fibroblastic, epithelial, endothelial, and muscle cells of the human intestinal tract and is activated in inflamed and fibrotic CD biopsies, making it an important hallmark of IBD-associated intestinal fibrosis.

Second, we showed that inhibiting ROCK activity by administration of AMA0825 in experimental murine models of intestinal fibrosis prevented accumulation of fibrotic tissue. We chose animal models based on a comparative analysis of ECM deposition in different models of IBD, including $TNF^{\Delta ARE}$ and A20 knockout mice, which exhibited only limited fibrosis (data not shown). The chronic DSS model and adoptive T-cell transfer model were selected based on the substantial accumulation of fibrosis, which was associated with increased intestinal ROCK activity. Systemic administration of ROCK inhibitors has been reported to result in anti-fibrotic effects in models of pulmonary, cardiac, hepatic, tubulointerstitial kidney, and retroperitoneal fibrosis, but it is associated with serious cardiovascular side-effects.[12,21,22] Use of the ROCK inhibitor, AMA0825, which is degraded upon contact with active esterases in the blood, has important advantages. It is well tolerated and does not cause cardiovascular hypotension in hypertensive rats, but it reaches the colon in concentrations well above the active range.

In our study, the anti-fibrotic effects were not accompanied by clinically relevant changes in inflammation, even though colonic IL6 was significantly reduced. This is surprising because IL6 is ubiquitously produced and broadly linked with inflammation. However, the serum concentrations of

IL6 in these mice did not decline, which provides further indication that the inhibitor was acting locally. Although other authors have described anti- inflammatory and barrier-protective effects of non-selective ROCK inhibitors (mainly Y27632),[16] in our study cytokine induction did not decrease in 3 in vivo models of intestinal inflammation, or in vitro in TNFα-challenged epithelial cells. AMA0825 was a stronger inhibitor of ROCK activity than Y27632 in intestinal mesenchymal cells (data not shown) and in epithelial cells. The reported anti- inflammatory actions of Y27632 may have been because of off-target effects of the compounds used, rather than to ROCK inhibition as such.[23]

Combining AMA0825 with anti-TNF in vivo not only ameliorated inflammation but also prevented accumulation of fibrotic tissue, underscoring the importance of combination therapy. These results indicate that combining anti- inflammatory agents with AMA0825 holds promise for the treatment of active, stricturing CD.

Additionally, using ex vivo cultures of biopsies from CD patients with fibrostenotic disease, we demonstrated that ROCK inhibition not only halted the release of pro-fibrotic mediators, but also specifically reduced the production of MMP-3 and -12. This is interesting because proteolytic degradation of anti-TNF monoclonal antibodies by locally produced MMP-3 and -12 leads to loss of responsiveness to anti-TNF therapy in CD patients.[24] Because AMA0825 reduced the ex vivo production of MMP-3 and -12 in ileal stenotic biopsies, it might enhance therapeutic responsiveness to anti-TNF. However, it must be noted that MMPs were quantified by Luminex technology, which does not assess their biological activities.

In a third set of experiments, we showed that AMA0825 reversed established fibrosis in vivo. Because up to 10% of CD patients already have a fibrostenosing phenotype at diagnosis, the possibility of reversal could diminish the prospects of intestinal surgery.[25]

Mechanistically, we showed that ROCK plays a central role in the generation of fibrosis effector cells, ie, intestinal smooth muscle cells, myofibroblasts, and mesenchymal cells derived from EMT. TGFβ1 in particular, which is excessively produced because of inflammation and contributes to wound healing, is a prominent inducer of the activation of smooth muscle cells and fibroblasts, and EMT as well. In primary fibroblast cultures, TGFβ1 induced formation of vimentin/actin-positive stress fibers, synthesis of collagen, and secretion of IL6, TGFβ1 and MMP. Blocking ROCK activity with AMA0825 prevented these pro-fibrotic events by averting the TGFβ1-induced activation of the MRTF (reducing the transcription of *MMPs*, *TGFβ1,* and *ACTA2*) and p38 MAPK (controlling IL6 release) signalling pathways,

but it was independent of SMAD signalling. In line with these observations, selectively blocking the MRTF pathway has been shown to inhibit the activation of colonic fibroblasts in response to TGFβ1 and matrix stiffness.[26] Because ROCK functions more upstream in the TGFβ1 pathway, it acts more broadly than selective MRTF inhibition and also interferes with myosin light chain phosphorylation and IL6 secretion, which are not controlled by MRTF. Indeed, we showed that p38 MAPK phosphorylation was also required for TGFβ1-induced IL6 secretion, and that AMA0825 inter- fered with this process. Although the roles of p38 and other MAPK pathway components in fibrosis are not fully understood, p38 MAPK and MEK/ERK signalling are generally considered pro-fibrotic. In HIFs, JNK did not play a part in IL6 release, whereas MEK1 inhibition sensitized cells to the effects of TGFβ1. Several drugs interfering directly with TGFβ1 activity have been evaluated in pre-clinical IBD models of fibrosis, but with limited success.[27–30] However, given the pivotal anti-inflammatory properties of TGFβ1, selective targeting of ROCK as a downstream mediator of TGFβ signalling holds advantages over general TGFβ inhibition.[31]

Inhibition of ROCK by AMA0825 enhanced autophagy in fibroblasts both in vitro and ex vivo and contributed to the decrease in collagen and IL6 production in response to TGFβ1. For collagen, this effect could have been because of increased intracellular degradation in autophagic bodies, as reported by others for atrial fibroblasts.[32] Furthermore, inhibition of autophagy affected IL6 protein secretion as well as transcription, pointing to the existence of an upstream factor that is targeted for degradation. Because p38 MAPK controls IL6 release from HIFs, and inhibition of autophagy increases the level of phospho-p38 MAPK in the cell, this could represent a link between IL6 and autophagy. However, whether p38 MAPK itself is targeted for autophagosomal degradation is not known. Alternatively, p62, which is targeted to autophagic bodies and subsequently degraded, is a known TGFβ-independent activator of the p38 MAPK pathway.[33] Therefore, the down-regulating effects of ROCK inhibition on p38 MAPK phosphorylation may be connected to increased levels of autophagy. Similarly, cross-talk between p38 MAPK and ROCK has been described in lung endothelial cells, in which Y 27632 could suppress p38 MAPK activation upon bacterial ligand stimulation.[34]

In conclusion, we report the efficacy of a locally active, oral ROCK inhibitor that prevents and resolves intestinal fibrosis through reduction of fibroblast activation combined with an increase in the autophagic response in these cells. We recommend that this agent, AMA0825, be evaluated further as a promising add-on therapy to existing anti- inflammatory agents for CD.

ACKNOWLEDGEMENTS

The authors wish to thank Katia Reynvoet and Sophie Vermaut (Dpt. of Clinical Chemistry, Microbiology and Immunology, UGent) for assisting with confocal microscopy and Elke Decrock (Dpt. of Basic Medical Sciences, UGent) for epifluorescence microscopy. Atg16L1$^{HM/HM}$ mice were kindly provided by Andy Wullaert (Inflammation Research Center, VIB and Dpt. Internal Medicine, UGent).

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SUPPLEMENTARY MATERIALS AND METHODS

Compounds and reagents. AMA0825 was designed, synthesized and profiled *in vitro* by Amakem Therapeutics (Diepenbeek, Belgium).[1] Recombinant TGFβ, CCG-1423, SP600125, PD0325901 and bafilomycin A1 were purchased from Sigma-Aldrich (Diegem, Belgium). SB203580 was acquired from Cell Signaling (Leiden, The Netherlands).

Pharmacokinetic profile of AMA0825 in mice. Ten-week-old male C57BL/6J mice received a single dose of AMA0825 in a mixture of propylene glycol (Sigma-Aldrich) and water (20/80 v/v) via oral gavage. Plasma and colon samples were collected 1, 2, 4, 8 and 24 hours after AMA0825 administration. At terminal endpoint, blood was sampled retro-orbitally under anesthesia (100 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine) and collected in tubes containing EDTA (5 mM final concentration). Plasma was extracted and samples were stored at -80 °C until analysis. Mice were sacrificed and their colons were excised, rinsed with ice cold saline, blotted dry, weighed, snap frozen and stored at -80 °C. The colon samples were subsequently homogenized, and the concentration of AMA0825 was determined by LC/MS-MS analysis.

Determination of cardiovascular side-effects. Sixteen-week-old Spontaneously Hypertensive rats (Charles River Laboratories) were anesthetized using isoflurane and were subcutaneously (s.c.) administered 7.5 mg kg^{-1} carprofen (Rimadyl®). Following a midline incision in the abdomen, a DSI TA11PA-C40 (Data Sciences International, s'Hertogenbosch, The Netherlands) implantable telemetric device was introduced into the peritoneal cavity, and the catheter of the device was inserted facing upstream into the descending aorta at a point below the renal arteries. The abdominal and skin incisions were then closed. The animals were given 100 mg kg^{-1} amoxicillin intramuscularly (i.m.) and returned individually to their cages. After 24 hours, they were given 100 mg kg^{-1} amoxicillin s.c. One week later, the animals were placed individually within their home cage on a telemetry receiver (Data Sciences International) to record mean, systolic and diastolic arterial blood pressures (mmHg), as well as heart rate (beats/min, derived from pulse blood pressure). All generated data were acquired and analyzed using EMKA Technologies software. AMA0825 was administered via oral gavage, and data were recorded continuously from 30 min before to 4 hours after the administration of AMA0825. Effects were reported at the following time points: 0, 15, 30, 45, 60, 120, 180 and 240 min after each application. Each animal received both vehicle and the test substance, with a washout period of at least 48 hours between each treatment.

Histology. Paraffin-embedded colon sections (4 µm thick) were deparaffinized, rehydrated with serial immersions in ethanol and stained with hematoxylin and eosin (Sigma-Aldrich). To visualize tissue fibrosis, Masson's trichrome staining was applied according to the manufacturer's protocol (Sigma-Aldrich). Inflammation was scored as previously described.[2] Fibrosis was quantified using a combined score of fibrosis severity (0=no fibrosis, 1=increased ECM deposition in the mucosa, 2=increased ECM deposition in the submucosa, 3=thickening of the muscularis mucosae, 4=thickening of the muscularis propria, 5=ECM deposition in the serosal layers), circularity (1=0 – 25%; 2=25 – 50%; 3=50 – 75%; 4=75 – 100%) and the extent of fibrosis (1=distal colon; 2=distal and mid colon; 3=full length of the colon). The final scoring was represented as the mean of the scores determined by TH and an expert pathologist (KG) (Cronbach's α =.78), who were both blinded to the samples.

ROCK activity assay. ROCK enzymatic activity was determined using a commercially available enzyme immunoassay (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, tissue lysates were incubated for 1 hour on plates pre-coated with myosin phosphatase target subunit 1 containing a Thyr696 residue, which is phosphorylated by ROCK1/2. An antibody against phospho-Thyr696 was added, followed by an HRP-labeled detection antibody. After substrate addition, the signal was measured at 450 nm (Multiskan Ascent, VWR International, Leuven, Belgium). Data were normalized to the total protein content (Bio-Rad Laboratories, Temse, Belgium).

Myeloperoxidase activity. Distal colonic myeloperoxidase activity was determined as described previously.[3]

Immunohistochemistry. Paraffin-embedded colon sections (4 µm thick) were deparaffinized, rehydrated by serial immersion in ethanol and pretreated with a citrate buffer at 95 °C for 1 hour, followed by the blocking of endoperoxidase activity via incubation in 3% H_20_2 (Merck) for 10 min. Prior to the addition of the primary antibody (1/200 anti-αSMA (Dako, Glostrup, Denmark)) for 30 min at room temperature, mouse IgG was blocked for 1 hour at room temperature using the Mouse on Mouse kit (Dako). Detection was performed using the Vectastain ABC kit and a 3,3'-diaminobenzidine (DAB) substrate addition (all from Vector Laboratories, Burlingame, USA). F4/80 staining was performed on paraffin-embedded colon sections pretreated with an Antigen Retrieval solution (Dako). After blocking with 3% H_20_2 and 20% rabbit serum (Dako), the primary antibody (1/200 anti-F4/80 (Serotec, Dusseldorf, Germany)) was applied overnight at 4 °C, followed by detection using the Vectastain Elite ABC kit and a DAB addition (Vector Laboratories). A ROCK enzyme staining was performed on paraffin-imbedded ileal sections (4 µm thick). Slides were pretreated with Tris/EDTA (pH 9) at 95°C for 1 hour

and then underwent blocking with 3% H_20_2 and 10% goat serum (Dako), followed by incubation with the primary antibody (1/200 rabbit anti-ROCK1 (Abcam)) overnight at 4 °C. For detection, a secondary polyclonal HRP-conjugated goat anti-rabbit antibody was used, followed by detection using the Vectastain Elite ABC kit and DAB (Vector Laboratories). Slides were counterstained with hematoxylin and eosin. Computerized semi-quantitative analyses were performed using Cell D software (Olympus Imaging Solutions, Munster, Germany).

RNA extraction. Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) with on-column DNase treatment. The concentration and purity of the RNA was determined using NanoDrop technology (Eppendorf, Rotselaar, Belgium). All samples exhibited an OD260/OD280 ratio between 1.8 and 2.1.

Quantitative real-time PCR. One microgram of total RNA was converted to single stranded cDNA via reverse transcription using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Fifteen ng was used for quantitative real-time PCR (qRT-PCR) using SYBR Green (GC biotech, Alphen a/d Rijn, The Netherlands) and 250 nM of each primer. A two-step program was performed on a LightCycler 480 system (Roche, Vilvoorde, Belgium). Cycling conditions were 95 °C for 10 min, 45 cycles of 95 °C for 10 sec and 60 °C for 1 min. A melting-curve analysis was used to confirm primer specificities. All reactions were performed in duplicate. The PCR efficiency of each primer pair was calculated using a standard curve of reference cDNA. Amplification efficiency was determined using the formula $10^{-1/slope}$, and primer pairs were selected based on an efficiency of 90-110%. Primer pair sequences and their efficiencies are listed in Table 2. Expression data were calculated relative to the mean of the overall expression level and normalized to the median expression of the stably expressed reference genes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex A subunit (SADH) and hydroxymethylbilane synthase (HMBS) (expressed as normalized relative quantities, NRQs).

Cytokine and MMP quantification. Protein levels of IL6, IL8, IFNγ, TNFα, MCP1, CXCL1, TGFβ1 and MMPs in the supernatant and tissue samples were determined using Luminex technology according to the manufacturer's guidelines (Bio-Rad Laboratories). Data from tissue samples were normalized to the total protein content (Bio-Rad Laboratories).

Western blot analysis. Cells were lysed in a radio immunoprecipitation assay (RIPA) buffer supplemented with phosphatase and a protease inhibitor cocktail (Sigma-Aldrich). The concentrations of protein lysates were determined (Bio-Rad Laboratories), and 30 μg of each sample was separated

on a 4-20% Criterion Stain Free gradient gel (Bio-Rad Laboratories). Next, the gel was activated by UV exposure for 1 min using the Chemidoc MP Imaging system (Bio-Rad Laboratories), and proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked with 5% skim milk in Tris buffered saline with 0.1% Tween-20 (TBST) (Sigma-Aldrich) and incubated overnight at 4 °C with primary antibodies (1/1,000 dilution) in 5% BSA/TBST (anti-phospho p38 MAPK (Cell Signaling), anti-p38 MAPK (Cell Signaling), anti-LC3B (Sigma-Aldrich), anti-p62 (Sigma-Aldrich), and anti-MRTFA (Cell Signaling)). Next, blots were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies (1/10,000 dilution, Cell Signaling). Bands were visualized using chemiluminescence (Bio-Rad Laboratories) and imaged on a Chemidoc MP Imager. Band intensities and total protein were quantified and analyzed using ImageLab 5.2 software.

***In vitro* assays.** Human intestinal fibroblasts were seeded at a density of 5,000 cells/cm^2, using a passage number between 3-5. The next day, cells were stimulated with 1 ng/mL TGFβ1 for 48 hours in the presence of a range of doses AMA0825 (0.001 nM to 100 µM, 10-fold dilutions) or the vehicle control. Supernatants samples were collected for cytokine, MMP and lactate dehydrogenase (LDH) quantification (Biovision, California, USA). Cells were incubated for an additional 3 hours with 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich). The remaining supernatant was discarded, and the MTT precipitate was dissolved in dimethyl sulfoxide (DMSO) and measured at 570 nm (Multiskan Ascent). This experiment was repeated in the presence of 10 nM bafilomycin A1, 10 µM CCG-1423, 10 µM SB431542, 10 µM SB203580, 20 µM SP600125 or 10 µM PD98059. Supernatants were collected and cells were harvested for protein and RNA isolation.

HT29 epithelial cells and human ileal endothelial cells (HIMECs) were stimulated with respectively 100 ng/ml TNFα + 300 ng/mL IFNγ or 100 ng/mL TNFα for 24 hours in the presence of a dose range of AMA0825 (0.01 nM to 100 µM, 10 fold dilutions). Supernatant was collected for cytokine and LDH measurement.

For the TEER experiments, Caco-2 cells were seeded on 24-well semipermeable inserts (0.4 µm, translucent ThinCerts™, Greiner Bio-One) at a density of 100.000 cells per well. Cells were left to differentiate over the course of 2 to 3 weeks until functional monolayers with absolute TEER-values of more than 3000 Ohm were obtained. THP1 cells were seeded in 24-well plates (Greiner Bio-One) at a density of 500.000 cells/well and treated with 50 ng/ml of PMA for 48 hrs. After 48 hrs, adherent THP1 cells were washed once with PBS after which the wells were filled with Caco-2 culture medium. Then, the Caco-2 inserts were placed on top of the PMA-differentiated THP1 cells. Next, the Caco-2 monolayers were stimulated apically with a dose range of AMA0825 (0.08 µM to 50 µM, 5-fold dilution)

or 5 mM butyric acid as positive control. After 48 hrs, absolute TEER values were normalized to their pre-treatment values and expressed as percentage of the initial resistance. Each condition was performed in triplicate.

Immunofluorescence. Human intestinal fibroblasts were grown on culture slides (Becton Dickinson, Erembodegem, Belgium), washed with ice cold PBS, fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton for 1 hour followed by incubation with anti-vimentin (1/1,000 dilution, Cell Signaling) for 1 hour at room temperature. Next, cells were incubated for 30 min with an Alexa-Fluor-labeled secondary antibody (1/1,000 dilution, Life Technologies). Actin stress fibers were visualized using CytoPainter Phalloidin-iFluor (Abcam, Cambridge, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies). Inserts were mounted and visualized using epifluorescence microscopy (Olympus BX61, Olympus, Berchem, Belgium). ImageJ software was used for the quantification of the fluorescent signals.[4]

Transmission electron microscopy. Human intestinal fibroblasts cell cultures grown on glass cover slips were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in a 0.1 M Na cacodylate buffer (pH 7.2) for 4 hours at room temperature, followed by fixation overnight at 4 °C. After washing, cells were subsequently dehydrated in a graded ethanol series, including a bulk staining with 1% uranyl acetate at the 50% ethanol step, and were then embedded in Spurr's resin. Ultrathin sections with a gold interference color were cut using an ultramicrotome (Leica EM UC6), followed by post-staining in a Leica EM AC20 system for 40 min in uranyl acetate at 20 °C and for 10 min in lead stain at 20 °C. Sections were collected on formvar-coated copper slot grids. Grids were viewed with a transmission electron microscope (Jeol JEM1010; Jeol, Tokyo, Japan) operating at 60 kV. Autophagosomes were counted in 10 cells per condition, 4 images per cell (magnification: 5,000x).

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SUPPLEMENTARY TABLES

Supplementary Table 1: Patient characteristics

	Inflammatory CD biopsies	Fibrostenotic CD biopsies	Fibrostenotic CD resection	Healthy subject resection
N	5	8	6	2
Mean Age in yrs (range)	34 (12-58)	48,5 (38-71)	46 (32-51)	50 (49-51)
Disease duration in yrs (range)	3 (3-6)	13 (2-27)	14,5 (5-18)	NA
Anti-TNF use	2/5 (33%)	4/8 (50%)	4/6 (67%)	NA

NA=not applicable

Supplementary Table 2: Primer pairs used for qRT-PCR

Gene symbol	Species	Forward (5'-3')	Reverse (5'-3')	E (%)
COL1A1	human	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC	98
MMP2	human	TGATCTTGACCAGAATACCATCGA	GGCTTGCGAGGGAAGAAGTT	106
ACTA2	human	AAAAGACAGCTACGTGGGTGA	GCCATGTTCTATCGGGTACTTC	106
TGFB1	human	CGACTACTACGCCAAGGAGG	CGGAGCTCTGATGTGTTGAA	96
IL6	human	GGCACTGGCAGAAAACAACC	GCAAGTCTCCTCATTGAAGCC	104
GAPDH	human	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	91
SDHA	human	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	92
HMBS	human	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	101
Col1a1	mouse	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG	105
Gapdh	mouse	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA	88
Hmbs	mouse	AAGGGCTTTTCTGAGGCACC	AGTTGCCCATCTTTCATCACTG	95
Sdha	Mouse	CTTGAATGAGGCTGACTGTG	ATCACATAAGCTGGTCCTGT	102

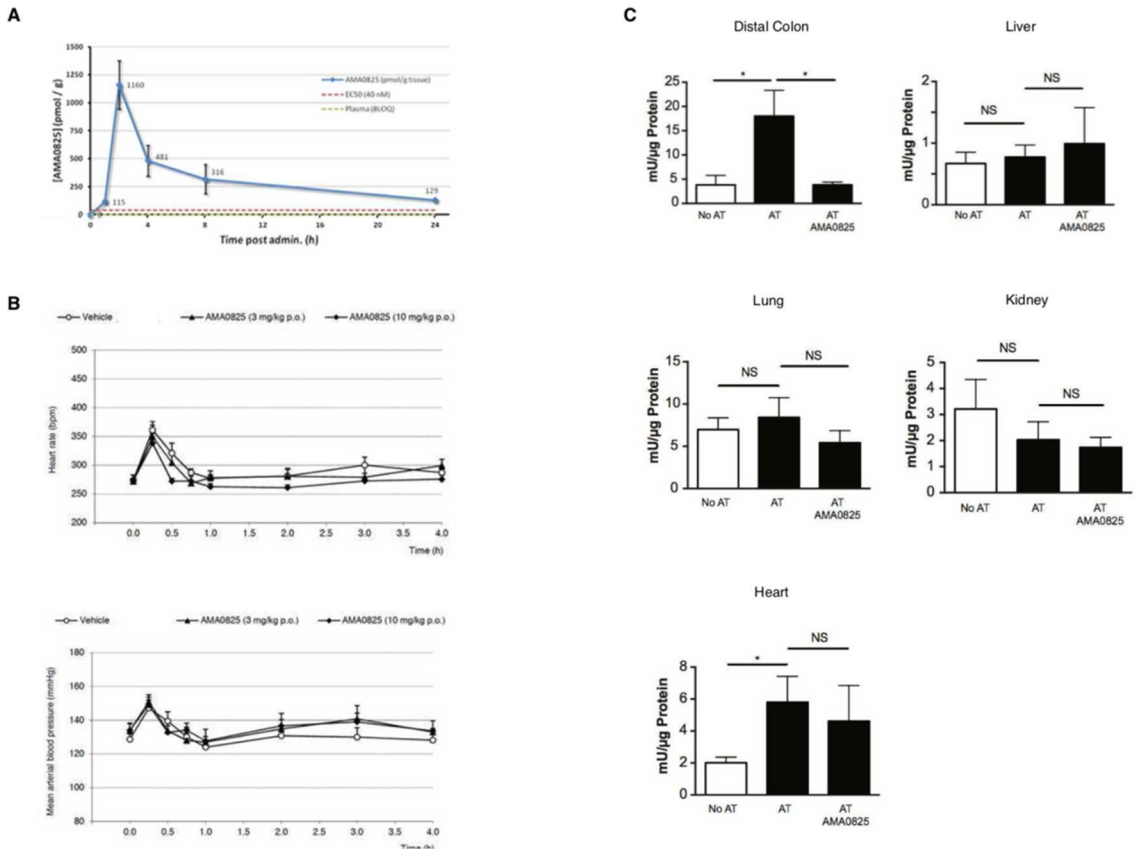
E=amplification efficiency

Supplementary Table 3: Effect of AMA0825 treatment on MMP expression in fibrotic tissue

	Chronic DSS prophylactic	Chronic DSS therapeutic	Intestinal fibroblasts	Stenotic biopsy cultures
MMP2	↓**	↓*	↓**	↓*
MMP3	↓*	NS	NS	↓*
MMP7	NA	NA	NA	NA
MMP8	NS	↓**	NA	NA
MMP9	↓*	↓*	NA	NS
MMP12	NS	↓**	↓**	↓*
MMP13	NA	NA	NA	NS

**P*<.05; ***P*<.01; NS=not significant; NA=not applicable, i.e., not different between healthy and fibrotic samples

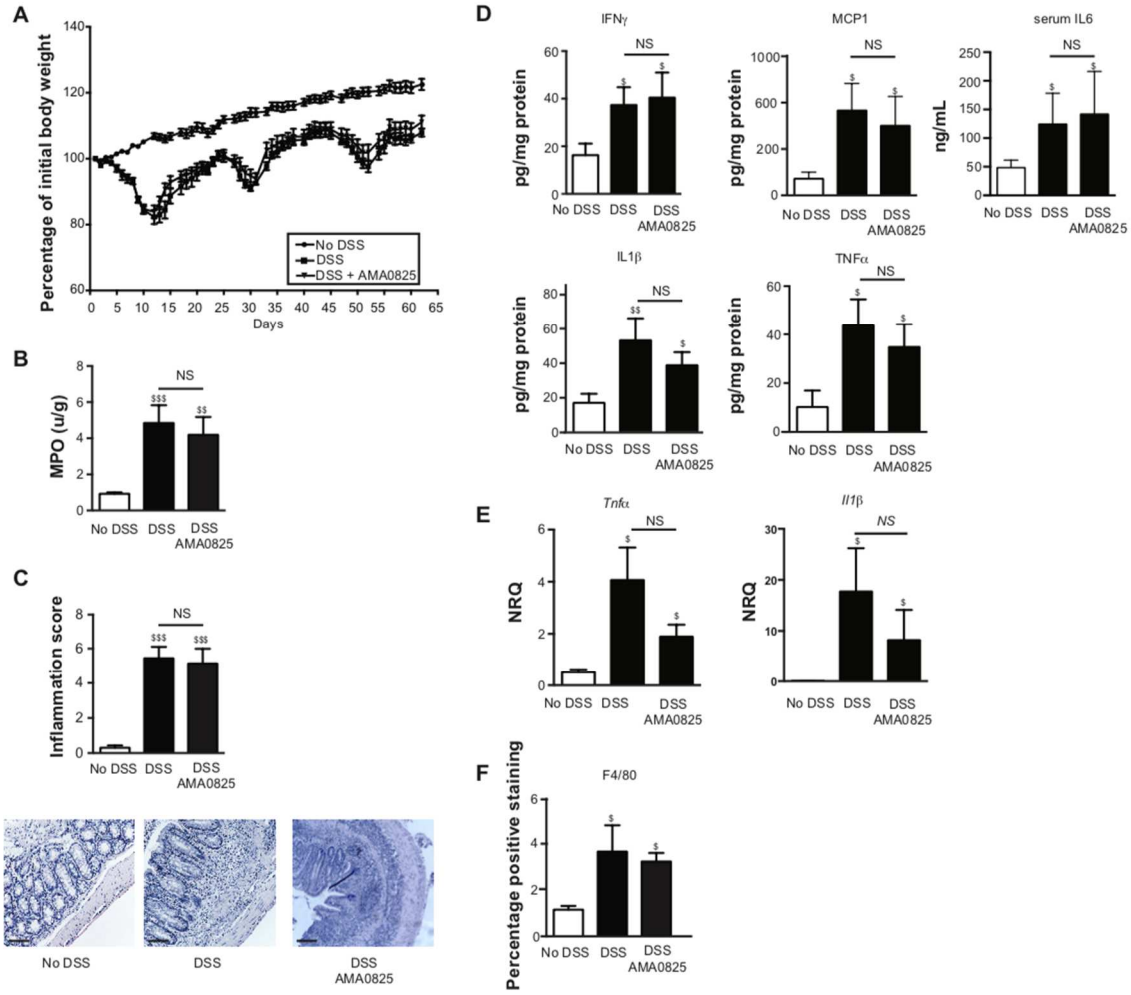
SUPPLEMENTARY DATA



SUPPLEMENTARY FIGURE 1 – Pharmacokinetic profiling of AMA0825 and effects on ROCK activity in different organs.

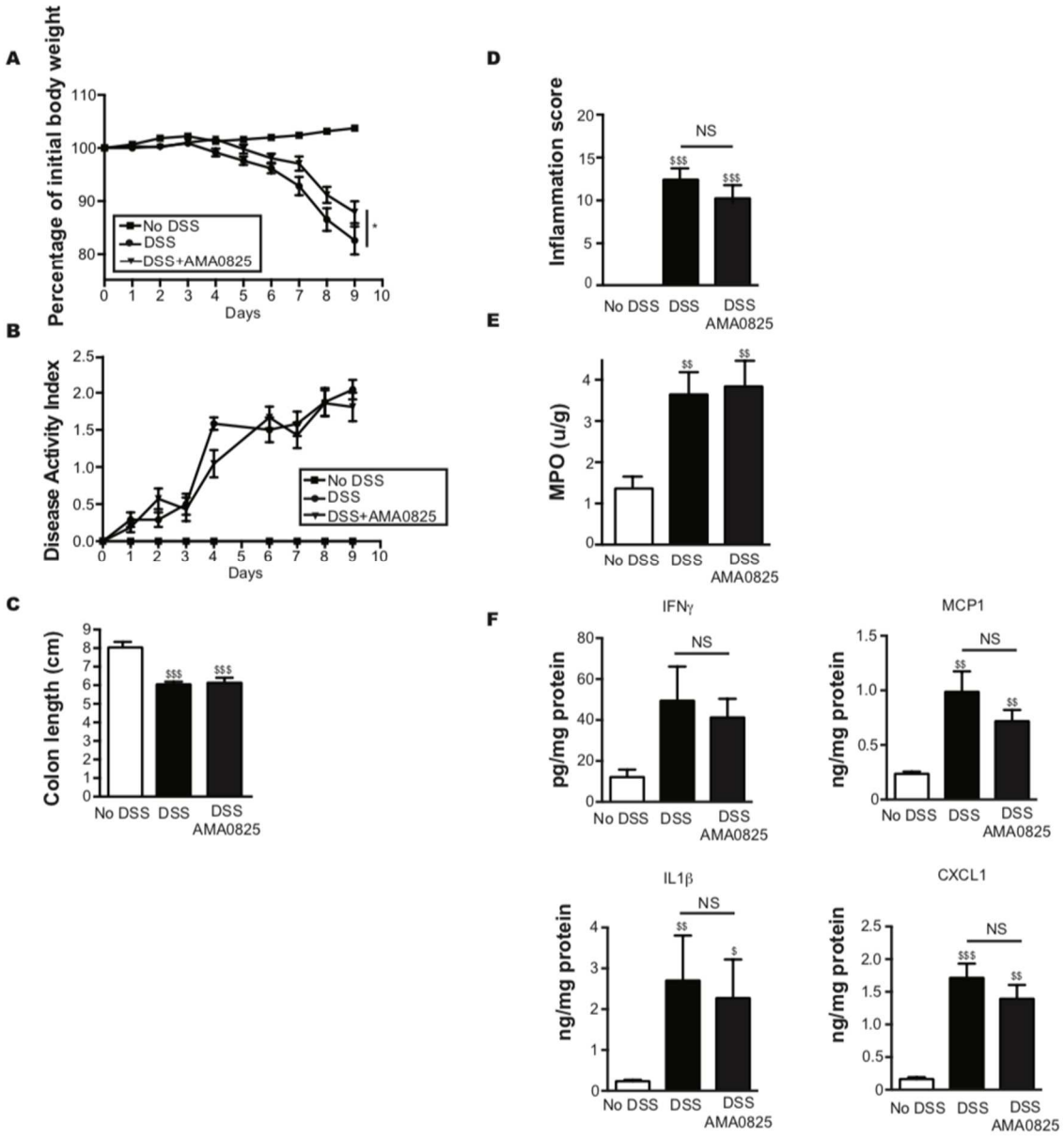
(A) The pharmacokinetic profile of AMA0825 was evaluated in ten-week-old C57BL/6J mice by administering 3 mg/kg via oral gavage. Plasma and colon samples were collected 1, 2, 4, 8 and 24 h after administration and AMA0825 was determined by LC/MS-MS analysis. From one h after administration, the plasma concentrations were below the level of detection (1 ng/ml), while concentrations in the colon were still >150 nM after 24 h, corresponding to approximately three times the EC50 (previously determined by an MLC phosphorylation assay). (B) Heart rate and blood pressure were monitored in spontaneous hypertensive rats after administration of a single oral dose of AMA0825 (3 and 10 mg/kg). Inter-group comparisons were performed (each test substance compared separately) using a two-way analysis of variance (group, time), with repeated measures at each time, followed by a one-way analysis of variance (group) at each time point in cases where there was a significant group x time interaction. AMA0825 at 3 and 10 mg/kg did not significantly modify arterial blood pressure or heart rate compared with the effect of the vehicle control. (C) ROCK activity in different organs during adoptive T cell transfer in AMA0825- and placebo-treated mice. To induce colitis, CD4+CD25-CD62L+ naive T cells were injected IP in CB-17 SCID mice. Mice developed symptoms of colitis from week 2 onwards, at which point therapy was initiated. Mice were treated with 3 mg/kg AMA0825per day or vehicle via oral gavage. Mice were sacrificed 45 days post- adoptive T cell transfer

and ROCK activity was measured in full lysates from the distal colon, liver, lung, kidney and heart. In the distal colon, adoptive transfer induced ROCK activity which was diminished significantly by AMA0825 administration. Rho kinase activity in the liver, lung and kidney was not induced by adoptive T cell transfer, while adoptive transfer did stimulate ROCK activity in cardiac tissue. In none of these organs AMA0825 significantly reduced ROCK activity, indicating a localized action of the inhibitor. n=10 in each group. AT: adoptive transfer. *P<.05, NS=not significant.



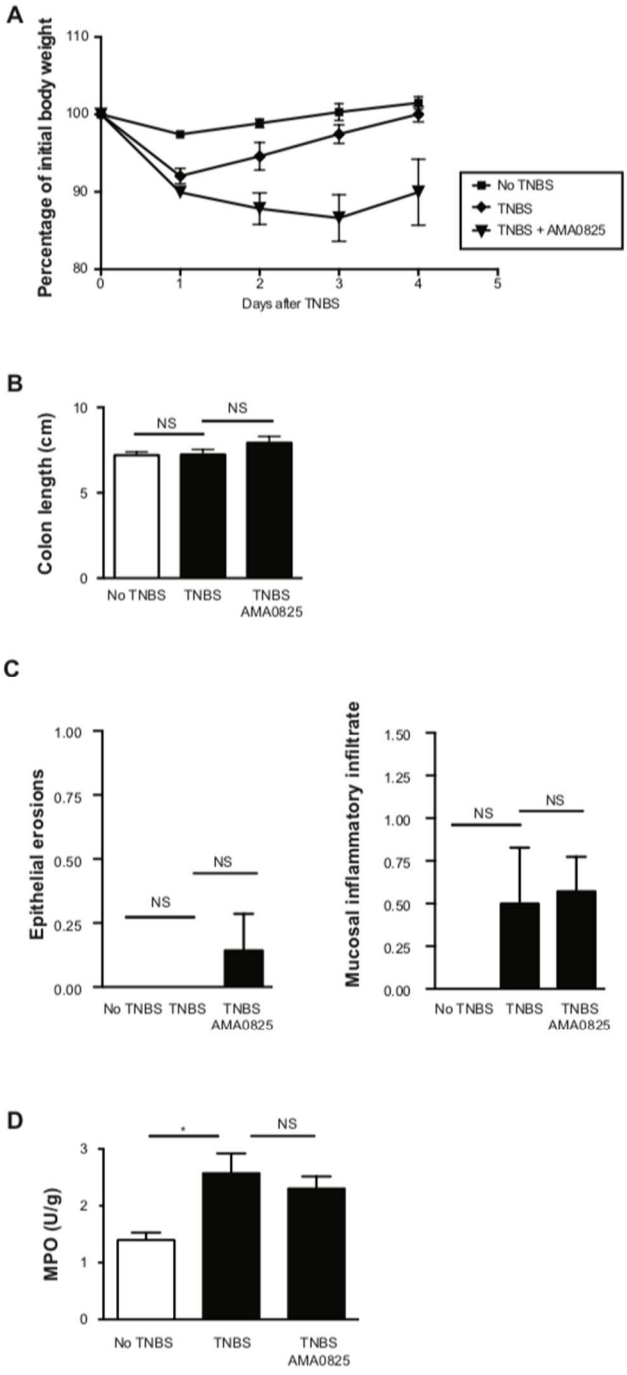
SUPPLEMENTARY FIGURE 2 - Preventive treatment with AMA0825 does not reduce inflammation during chronic DSS-induced colitis. C57BL/6J mice were subjected to three cycles of 2.5% DSS administration for one week followed by two weeks of recuperation. Mice were treated with 3 mg/kg/d AMA0825 from the start of DSS administration by oral gavage (n=19 in each group, pooled from two independent experiments). (A) Weight evolution, (B) colonic myeloperoxidase (MPO) activity, and (C) histological inflammation scores of the distal colon with representative H&E images (200x). (D) Colonic protein levels of IFNγ, MCP1, IL1β, TNFα and serum IL6 measured by Luminex

magnetic beads technology. (E) Quantification of F4/80 staining in the colon. Data are represented as mean±SEM. $ P<.05, $$ P<.01, $$$ P<.001 compared with the No DSS group. NS=not significant.

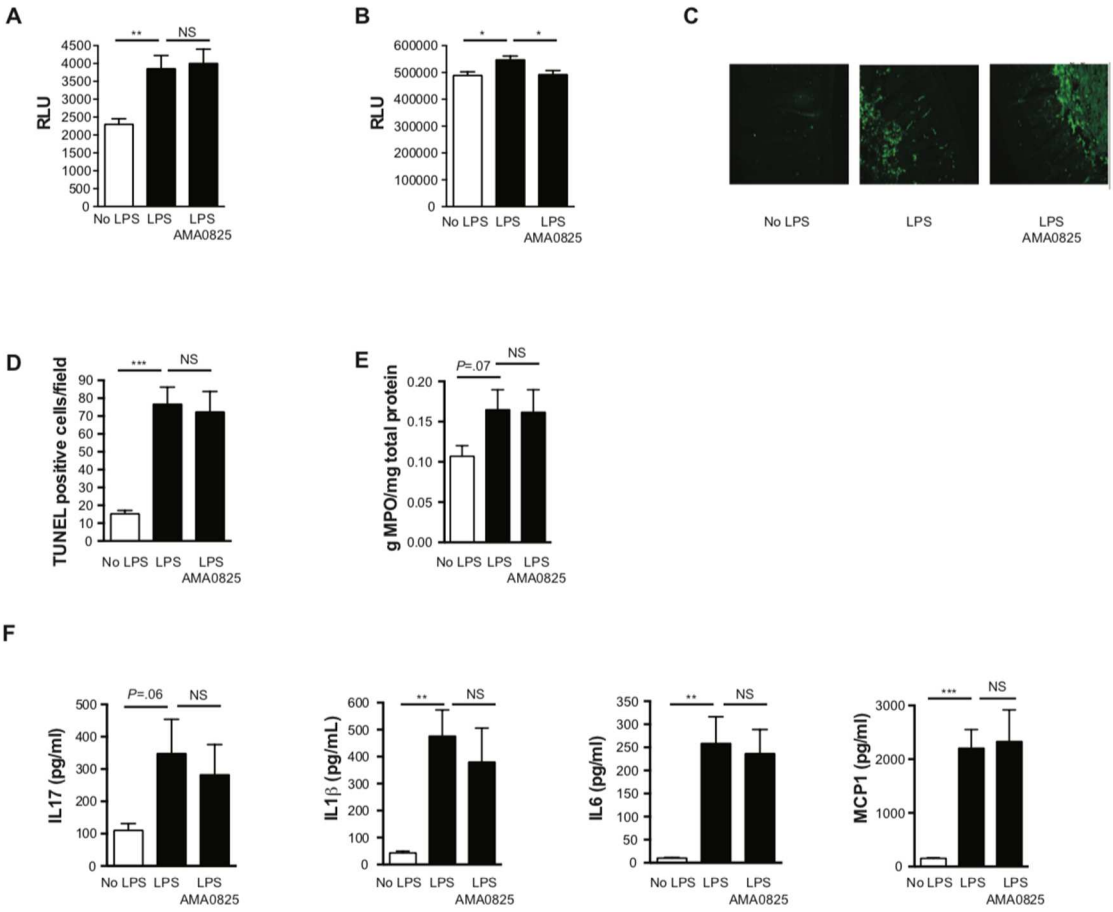


SUPPLEMENTARY FIGURE 3 - AMA0825 improves body weight evolution but does not reduce other parameters of inflammation in acute DSS-induced colitis.

C57BL/6J mice were subjected to 4% DSS administration for one week followed by 2 days of normal drinking water. Mice were treated with 3 mg/kg/d AMA0825 from the start of DSS administration by oral gavage (n=8 in each group). (A) Weight evolution, (B) disease activity index, (C) colon length, (D) histological inflammation scores, (E) colonic myeloperoxidase (MPO) activity levels, (F) colonic protein concentrations of IFNγ, MCP1, IL1β and CXCL1 measured by Luminex magnetic bead technology. Data are represented as mean±SEM. *P<.05; $ P<.05, $$ P<.01 compared with the No DSS group.

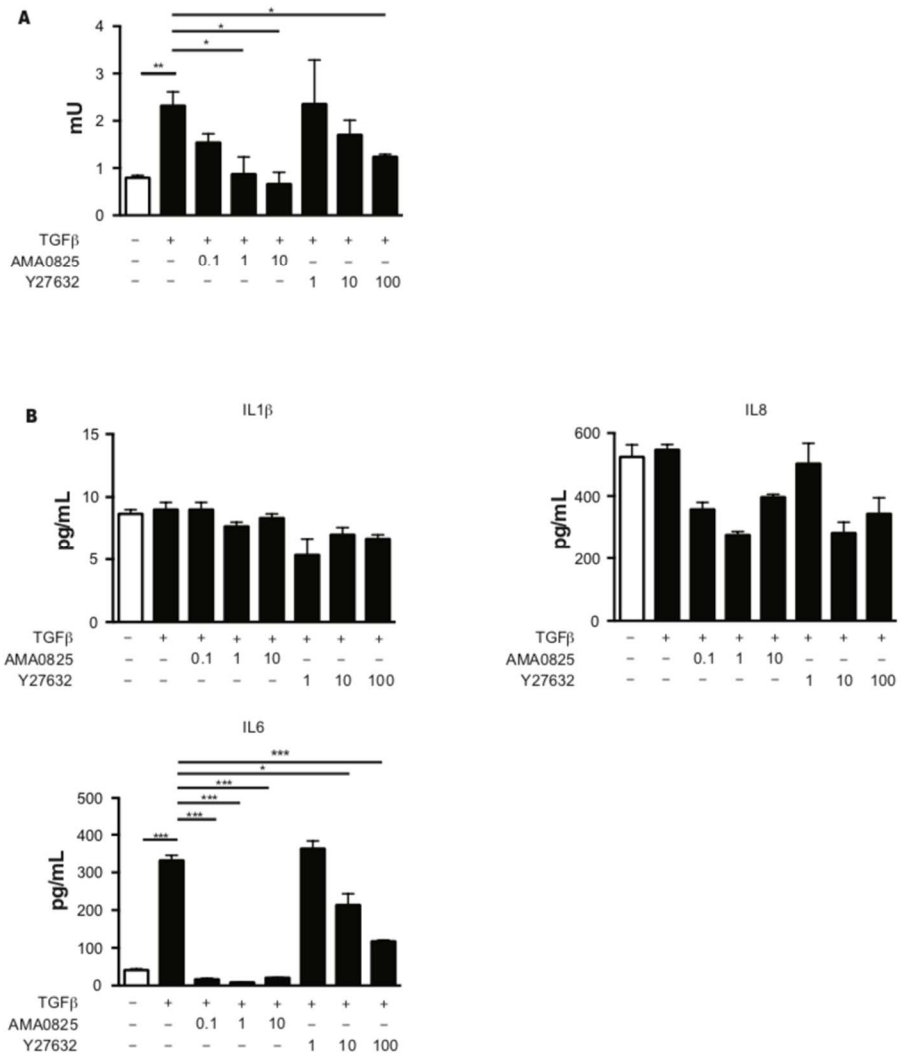


SUPPLEMENTARY FIGURE 4 - AMA0825 administration improves body weight evolution but not histological inflammation in a TNBS model of acute colitis. (A) Weight loss, (B) colon length, (C) scores for epithelial erosions and mucosal influx of inflammatory cells in distal colonic histological sections, and (D) MPO activity in full-thickness distal colonic lysates. Data represent the mean ± SEM of 7 or 5 mice/group. *P < .05; NS, not significant.

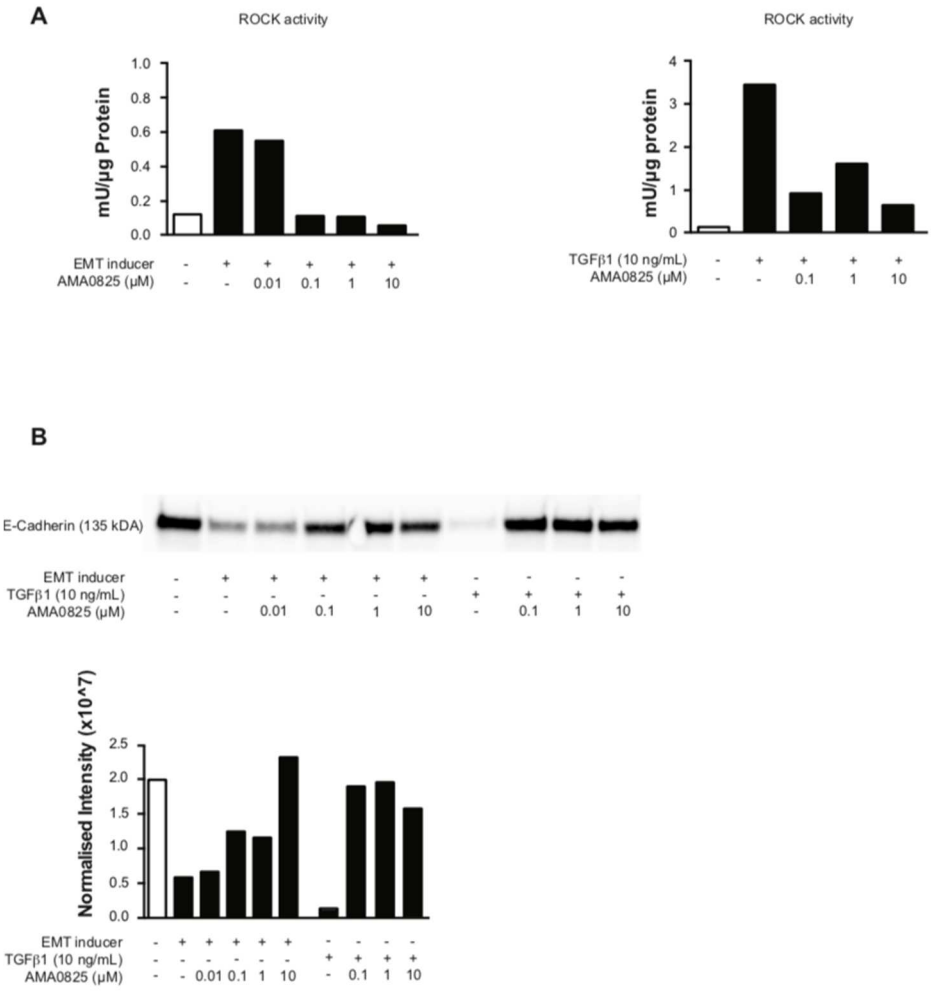


SUPPLEMENTARY FIGURE 5 - Effect of AMA0825 on LPS-induced intestinal permeability and enterocyte apoptosis

(A) FITC-dextran fluorescence in plasma, (B) caspase-3/7 activity in full-thickness ileal lysates, (C) representative images (400x) of TUNEL-stained sections of the terminal ileum of non-LPS control mice and mice treated with LPS and AMA0825 (3 mg/kg) (D) TUNEL-positive ileal enterocytes quantified in six fields per mouse (x400), (E) MPO activity in full-tickness ileal lysates, (F) IL17, IL1β, IL6 and MCP1 protein levels in full-thicknes ileal lysates as determined by Luminex magnetic bead technoogy. Data represent the mean ± SEM of 8 mice/group. *P < .05; ** P<0.01, *** P<.001, NS, not significant.

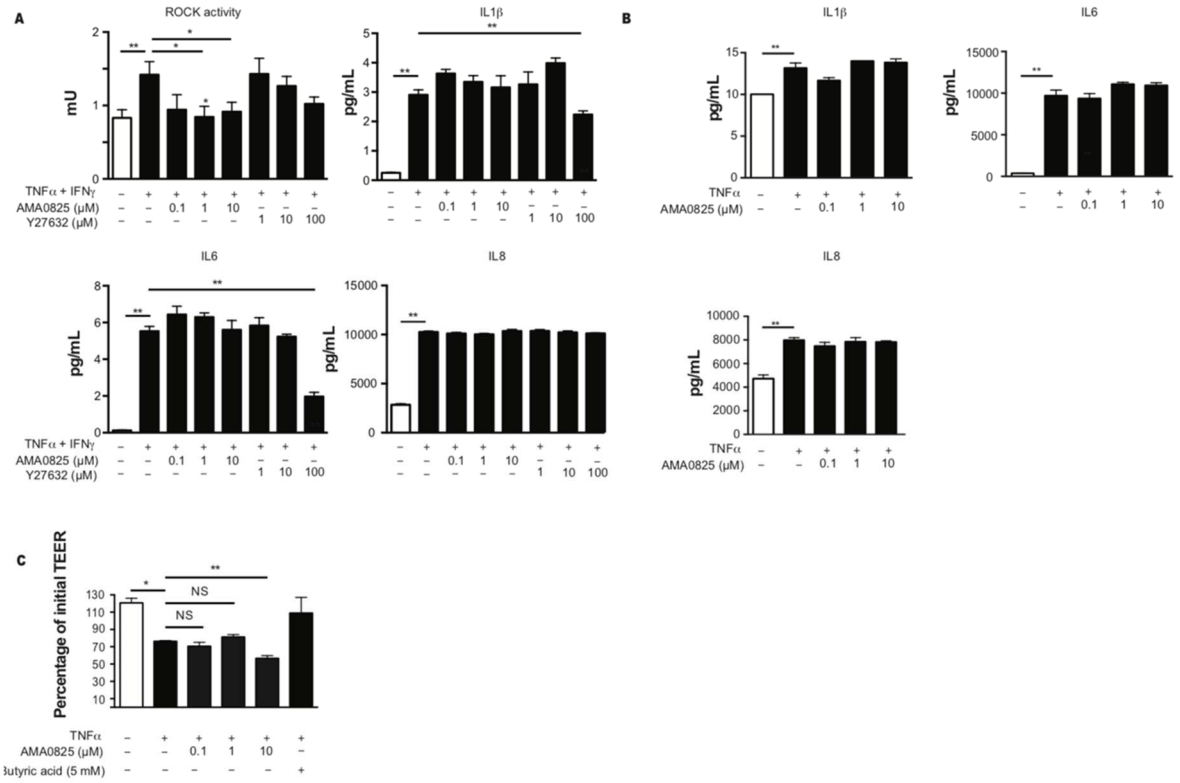


SUPPLEMENTARY FIGURE 6 - Effects of AMA0825 on intestinal smooth muscle cells. Primary intestinal smooth muscle cells (SMC, ScienCell) were seeded at a density of 5000 cells/ cm2 using a passage number between 3-5 for all experiments. The next day, cells were stimulated with 1 ng/mL TGFβ for 48 hours in the presence or absence of a dose range of AMA0825 or Y27632. Supernatant was collected for cytokine measurement, cells were harvested for ROCK activity assay. (A) ROCK activity (B) IL1β, IL8 and IL6 protein levels determined by Luminex magnetic bead technology. *P<.05, ** P<.01, *** P<.001



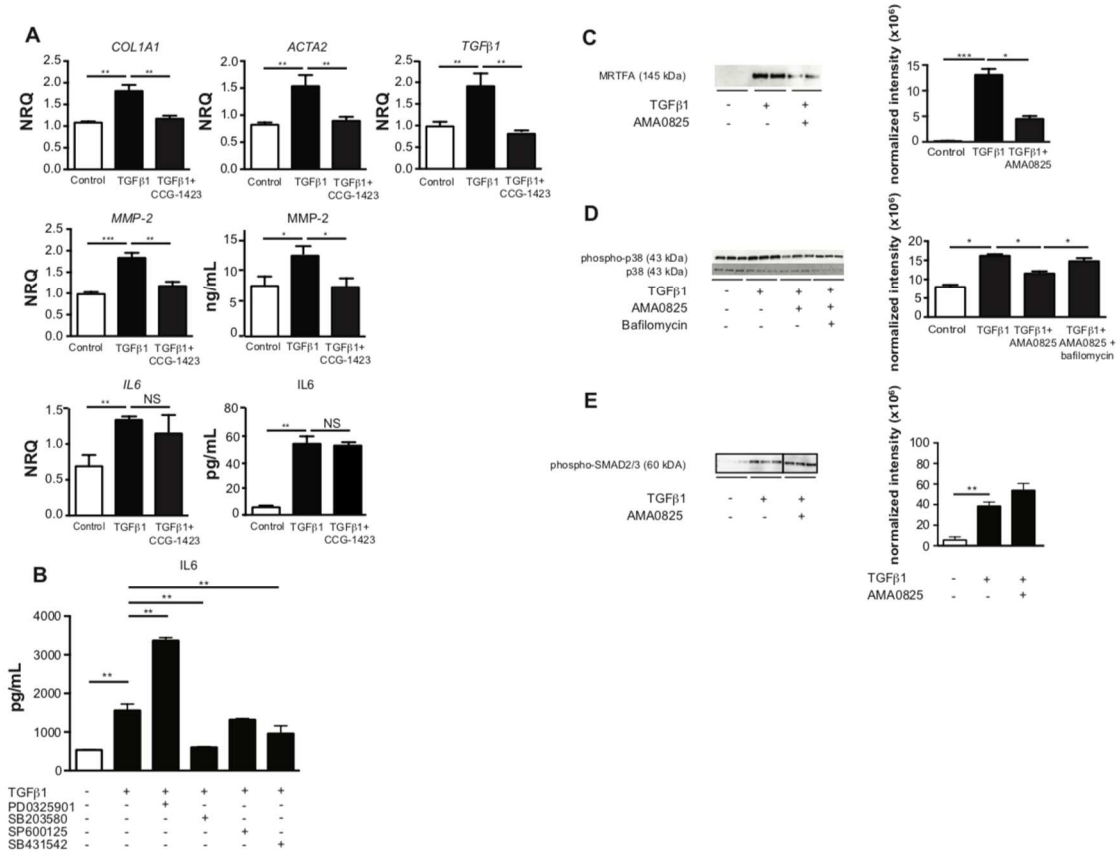
SUPPLEMENTARY FIGURE 7 - AMA0825 blocks epithelial-to-mesenchymal transition

HT29 were seeded at 150.000 cells/cm2 and stimulated the next day with an EMT inducing agent (a combination of recombinant Wnt5a, TGFβ1, anti-human E-cadherin, anti-human sFRP-1 and anti-human Dkk-1) in presence or absence of a dose range AMA0825 (10 µM – 0.01 µM) for 7 days with changing of the media every 2 days. Additionally, high dose TGFβ1 (10 ng/mL) was also used to induce EMT. Morphologic changes in the cells were followed by light microscopy at regular intervals. At day 7, cells were harvested for protein lysates and used for western blot and ROCK activity detection. (A) Rho kinase activity measured by immunoassay in HT29 lysates (B) Western blot images representing E-cadherin expression.



SUPPLEMENTARY FIGURE 8 - Effects of AMA0825 on other intestinal cell types

(A) HT29 cells were seeded at 150.000 cells/cm2 and stimulated the next day with a combination of TNFα 100 ng/mL and IFNγ 300 ng/mL for 24 hours in the presence or absence of a dose range of AMA0825. Supernatant was collected for cytokine detection, cells were harvested for Rho kinase activity measure- ment.(B) Human intestinal endothelial cells (HIMECs) were seeded at 100.000 cells/cm2 and stimulated the next day with TNFα 100 ng/mL for 24 hours in the presence or absence of a dose range of AMA0825. Supernatant was collected for cytokine detection, cells were harvested for Rho kinase activity measurement. (C) Caco-2 monolayers were stimulated basolaterally with a combination of TNFα and IFNγ, and treated apically with a dose range of AMA0825 (0.1 to 10 μ M, 10-fold dilution) or butyric acid (5 mM). TEER was measured 48 hrs post-stimulation and expressed as a percentage of the initial resistance prior to stimulation.



SUPPLEMENTARY FIGURE 9 - AMA0825 interferes with MRTF1 and p38 signaling in human intestinal fibroblasts but not SMAD2/3

Human intestinal fibroblasts (HIFs) were seeded at 5000 cell/cm2 and stimulated the next day with TGFβ1 1 ng/mL for 48 hours in the presence or absence of AMA0825, CCG-1423 (a MRTF1 inhibitor), PD0325901 (MEK1 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) or SB431542 (SMAD2/3 inhibitor). Cells were harvested for protein lysates and RNA isolation (A) mRNA transcript levels for COL1A1, ACTA2, TGFβ1, MMP-2 and IL6; concentration of MMP-2 and IL6 determined in the supernatant by luminex magnetic bead technology (B) IL6 concentration in the supernatant determined by luminex magnetic bead technology. Western blot analysis for (C) MRTF1 transcription factor (D) phopho-p38 and p38 and (E) phosho-SMAD2/3. Data represent the mean ± SEM of 3 replicates/group. *P<.05; **P <.01; ***P<.001.

II. Multi-locus genetic risk for early development of fibrostenosis in patients with Crohn's disease

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Grant support:

TH and DL are paid by grants from the Research Foundation Flanders (N° 11S7416N and 1298213N).

Author contributions:

TH, MDV, DL, SV, PH, IC: study concept and design

TH, PB, ID, IC, DL: acquisition of data and analysis

TH, MDV, PH, IC, DL: writing of the manuscript

All authors: critical revision of the manuscript

Status

Manuscript submitted to Gut (IF 16.658)

ABSTRACT

Objective: Fibrostenosis is a complication of Crohn's disease (CD) occurring in at least 30% of patients. Attempts to identify genetic markers for fibrostenotic CD were hampered by poor and subjective characterization of the study population. The aim of this study was to identify genetic markers by focussing on early, well-defined fibrostenotic CD.

Design: In this multicenter, retrospective, nested case-control association study, early ileal fibrostenotic CD was defined based on computed tomography or magnetic resonance enterography and occurring within five years following diagnosis. The control cohort consisted of ileal CD patients without fibrostenotic or penetrating complications for a minimum of ten years. Associations were assessed using Immunochip, and positive associations were evaluated in a replication cohort.

Results: Based on logistic regression and multiple logistic regression correcting for disease location, eight SNPs reached the 10^{-5} significance level in the discovery cohort, and two could be validated in the replication cohort. Meta-analysis revealed nine associated loci, which were used to calculate a genetic risk score (GRS) for each individual. Using a cut-off based on the AUC (0.885) in the discovery cohort, a positive predictive value of 54% and a negative predictive value of 69% was reached to predict early fibrostenosis in the validation dataset. The time to development of fibrostenosis was significantly linked with the GRS, with a 5-year fibrostenosis rate of 38% in patients with a high GRS compared with 4% in the low GRS group.

Conclusion: This carefully phenotyped association study reveals an important genetic contribution to the early development of fibrostenosis in ileal CD.

Keywords: inflammatory bowel disease, fibrostenosis, genetic risk, Immunochip, MIS18 binding protein, cadherin 4, epidermal growth factor receptor

INTRODUCTION

Crohn's disease (CD) is a chronic disorder of the gastrointestinal tract characterized by episodes of relapsing-remitting inflammation, mainly affecting the ileum and colon. In at least one third of patients, these recurrent episodes of transmural inflammation lead to the deposition of extracellular matrix in the (sub)mucosa resulting in the development of intestinal strictures, luminal stenosis and organ failure.[1] To this day, no medical anti-fibrotic treatment is available. Apart from endoscopic balloon dilation, surgery is the only therapeutic option, with associated loss of viable intestinal tissue and an endoscopic recurrence rate in up to 70-80% of patients.[2,3]

The pathophysiology of intestinal fibrosis involves a complex interplay of different cell types including epithelial cells, intestinal fibroblasts and smooth muscle cells and is believed to be influenced by environmental, disease-related and genetic factors.[4] Although the genetics of inflammatory bowel disease has been investigated extensively, rigorous associations of genetic factors with fibrostenotic disease behaviour are lacking. In previous association studies, genes such as *NOD2*, *ATG16L1*, *IL23R, TNFSF15* and *MMP3* have been linked with stenotic complications, but these associations were undiscernible from ileal disease location.[5] The lack of a solid definition of fibrostenotic disease is an important contributor to this problem: the majority of studies were subanalyses of large genome-wide association studies using the Montreal classification to identify patients with fibrostenotic complications.[6] Although the Montreal classification is frequently used in large population studies, it is not sensitive, nor specific, and a high inter-observer disagreement exists for identifying fibrostenosis.[7]

The velocity by which symptomatic fibrostenosis develops differs strongly between patients. At diagnosis, only 10% of patients present with stenotic complications but the proportion steadily rises to 30% after ten years.[1,3] Why some patients develop fibrostenotic disease more rapidly than others is unknown, but the genetic background may partly explain this discrepancy.

We hypothesized that applying a more accurate definition of fibrostenotic disease may increase the specificity and validity of genetic associations. In addition, we assumed that the genetic risk may be more important in patients with early fibrostenosis. Since cross-sectional imaging techniques such as computed tomography (CT) and especially magnetic resonance (MR) enterography correlate well with fibrosis on histopathology, we aimed to identify genetic markers by focussing on early fibrostenotic disease in a well-phenotyped population based on CT/MR enterography.[8-12]

MATERIALS AND METHODS

Study design and patient selection. Crohn's disease was diagnosed based on clinical, endoscopic and histological criteria. In this multicentre, retrospective, nested case-control study, performed at the University Hospitals of Ghent and Leuven, all CT and MR enterography scans performed in CD patients with ileal or ileocolonic CD (Montreal L1 or L3) from 2002 to 2016 were reviewed. In the Ghent/Leuven discovery cohort, 3,024 CT or MR scans from 2,042 CD patients were examined for signs of fibrostenotic disease, defined as the presence of bowel wall thickening with luminal narrowing and/or prestenotic dilatation. Patients with early fibrostenosis were defined as those with at least one positive scan occurring within five years following diagnosis. Only patients with Immunochip data available were included.[13,14] The control cohort was selected from local biobanks from Ghent and Leuven, including CD patients (Montreal L1 or L3) without arguments (defined as the absence of clinical, endoscopic, radiological or surgical signs of complicated disease) for fibrostenotic or fistulizing disease for at least ten years of follow up. This study was approved by the ethical committees of the participating centres (Ghent: 2016/0761, and Leuven: B322201213950/S53684).

Replication cohort. The replication cohort was selected at the University Hospital of Liège based on the review of 563 CT/MR scans. This study was approved by the local ethical committee (2005/49 and B707201419637/2014/3).

Genotyping and quality control. The samples were previously genotyped by means of the Immunochip array version 1 (Illumina, San Diego, CA, USA), a genotyping platform for screening 195,806 single nucleotide polymorphisms (SNP) previously selected by an expert panel from association studies of immune-related disorders.[13,15] Quality control of samples and genotypes was performed as previously described.[13,14]

Statistical analysis. Genetic association analyses were performed using PLINK version 1.9. To calculate genetic association, logistic regression was performed, or multiple logistic regression correcting for disease location. The level of genome-wide statistical significance for this Immunochip was set at $P<10^{-5}$. In the replication cohort, we focused on those SNPs associated in the discovery cohort, and $P<0.05$ was considered statistically significant. Fixed effects meta-analysis was performed on the summary statistics for the multiple logistic regression correcting for disease location of both the discovery and replication dataset.

A weighted genetic risk score (GRS) was calculated for each individual using the Mangrove R package (R 3.4.2).[16] Weights included the risk allele frequency and effect size (OR) from the logistic regression analysis correcting for disease location in the discovery cohort.

Other statistical analyses were performed in IBM SPSS version 24 (IBM Corp, Armonk, NY, USA). A ROC curve from the discovery cohort was generated to calculate a cut-off GRS value for the prediction of the development of fibrostenosis in the validation cohort. A Kaplan-Meier survival curve was generated to evaluate the influence of the GRS on the time to development of fibrosis in the discovery dataset.

RESULTS

Patient phenotyping

Based on the CT/MR scans of 2,042 CD patients with ileal or ileocolonic disease, 446 patients (22%) were classified as fibrostenotic (Figure 1). In this selection, 112 patients (25%) were diagnosed with fibrostenosis within five years of their initial CD diagnosis, of whom 60 (54%) had Immunochip data and were included (population characteristics are summarized in Table 1). No DNA was readily available from the remaining 52 patients. To illustrate the strength of the manifestation of fibrostenotic disease in this selected cohort, the diagnosis of fibrostenosis was based solely on CT/MR in 6 (10%) patients, 49 (82%) patients underwent a surgical resection of a stenosis with confirmation of fibrosis on histopathology, and stricturoplasty was performed in 5 (8%) patients. Of the 67 (15%) patients who developed fibrostenosis between six and ten years following CD diagnosis, 38 (57%) had Immunochip data and were additionally evaluated.

Age at CD diagnosis, gender distribution and previous tumor necrosis factor alpha antagonist exposure were similar between fibrostenotic and the non-fibrostenotic control group (Table 1). A statistically significant predominance of isolated ileal disease (Montreal L1) was present in the patients assigned to the fibrostenosis group (30/60 (50%) versus 131/343 (38%), P=0.02). The median interval between CD diagnosis and documentation of the fibrostenotic complication was 13.5 months (ranging between 0 and 60 months), and fibrostenotic disease was recognized already at diagnosis in 18 (30%) patients.

Discovery cohort		
	Yes	No
CT/MR scans available between 2002-1016 from CD patients (Montreal L1/L3)	2,042	
→ Fibrostenotic disease	446 (22%)	1,596 (78%)
→ Within 5 years following diagnosis	112 (25%)	
→ Immunochip data available	60 (54%)	52 (46%)
→ Within 6 - 10 years following diagnosis	67 (15%)	
→ Immunochip data available	38 (57%)	29 (43%)

Validation cohort		
	Yes	No
CT/MR scans available between 2002-1016 from CD patients (Montreal L1/L3)	563	
→ Fibrostenotic disease	350 (62%)	223 (38%)
→ Within 5 years following diagnosis	192 (55%)	
→ Immunochip data available	76 (40%)	116 (60%)

Figure 1. Schematic overview of patient selection.

Risk variants associated with early fibrostenotic disease in the discovery cohort

Based on logistic regression, six variants reached the 10^{-5} significance level for association (Table 2). Because of the significant predominance of Montreal L1 disease location in the fibrostenosis group, association was retested using multiple logistic regression with disease location as covariate. Genome-wide significance for two SNPs was lost (both located at chromosome 21), and two novel associations reached the 10^{-5} threshold (Table 2).

Validation of associations in a replication cohort and meta-analysis

Using identical selection criteria, 76 cases and 116 controls were collected in a third academic hospital centre for replication (Figure 1). Patient characteristics were similar as in the discovery cohort, including a significantly higher proportion of patients with isolated ileal disease in the fibrostenosis group (Table 1). However, there was a significant difference in

Table 1. Patient characteristics

	Discovery cohort			Replication cohort		
Variable	**Cases**	**Controls**	**P-value**	**Cases**	**Controls**	**P-value**
Number of patients	60	343		76	116	
Arguments for stenosis Only CT/MR Stricturoplasty Pathology	 6 (10%) 5 (8%) 49 (82%)	NA		 14 (18%) 1 (1%) 61 (80%)	NA	
Age (yrs; median, range) At CD diagnosis At diagnosis of fibrostenosis	 24 (10-68) 26 (14-68)	25 (3-72)	NS	 33 (11-75) 34 (14-75)	24 (12-72)	NS
Gender (% male)	46%	41%	NS	42%	41%	NS
Previous anti-TNF exposure	16 (27%)	66 (19%)	NS	16 (21%)	47 (41%)	**0.01**
Montreal classification L1 (ileal) L3 (ileocolonic)	 30 (50%) 30 (50%)	 131 (38%) 212 (62%)	**0.02**	 53 (70%) 23 (30%)	 48 (41%) 68 (59%)	**0.001**
Time to fibrostenosis (months; median, range)	13.5 (0-60)	NA		12 (0-60)	NA	
Fibrostenosis at diagnosis	18 (30%)	NA		34 (45%)	NA	

CT: computed tomography; MR: magnetic resonance; NA: not applicable

Table 2. Summary of SNPs associated with fibrostenotic disease in the discovery and replication cohort, and in meta-analysis

				Discovery cohort			Discovery cohort			Replication cohort			Meta-analysis			Included in GRS
				Logistic			Multiple logistic			Multiple logistic			Multiple logistic			
Chr	SNP	Risk allele	Gene	OR	95%CI	P-value	OR	95%CI	P-value	OR	95%CI	P-value	OR	P-value	95%CI	
7	rs4947982	A	*EGFR*	2.54	1.66-3.87	1.59E-05	2.47	1.62-3.79	2.95E-05			>0.05	1.88	3.95E-05		yes
14	rs35223850	A	*MIS18BP1*	3.48	1.96-6.20	2.22E-05	3.35	1.84-6.11	8.06E-05			>0.05	2.69	9.72E-05		yes
19	rs17554931	T	*GPX4*	3.99	2.09-7.61	2.73E-05	4.15	2.14-8.04	2.53E-05			>0.05	2.46	1.19E-03		yes
20	rs4925207	C	*CDH4*	3.33	1.90-5.94	2.84E-05	3.29	1.86-5.83	4.35E-05			>0.05	2.62	4.04E-05		yes
21	rs9325636	T		2.40	1.59-3.62	3.19E-05	2.16	1.41-3.29	3,72E-04	1.54	1.00-2.40	5.26E-02	1.84	9.32E-05		yes
21	rs3827232	T	*UBASH3A*	2.39	1.56-3.67	6.37E-05	2.01	1.35-3.32	9,01E-04			>0.05	1.37	5.45E-02		no
18	rs9960012	G		2.80	1.64-4.75	1,47E-04	3.22	1.85-5.61	3.56E-05			>0.05	1.91	2.06E-03		yes
1	rs12072417	G		8.64	2.88-25.91	1,19E-04	10.15	3.32-31.95	7.41E-05	5.42	1.05-27.91	4.32E-02	8.26	1.06E-05		yes
14	rs74062913	T		2.64	1.54-4.52	3,90E-04	2.82	1.62-4.90	2,47E-04	2.60	1.38-4.90	3,16E-03	2.72	2.57E-06		yes
14	rs17106208	T	*LOC105370547*	2.46	1.47-4.09	5,69E-04	2.68	1.58-4.54	2,68E-04	2.15	1.17-3.94	1,35E-02	2.43	1.24E-05		yes
14	rs17106237	A	*LOC105370547*	2.46	1.48-4.10	5,44E-04	2.68	1.58-4.55	2,59E-04	2.13	1.16-3.91	1,49E-02	2.43	1.33E-05		no
14	rs17106213	A	*LOC105370547*	2.40	1.44-3.99	7,74E-04	2.63	1.55-4.47	3,39E-04	2.15	1.17-3.94	1,35E-02	2.41	1.53E-05		no
14	rs10143427	C	*LOC105370547*	2.40	1.44-3.99	7,74E-04	2.63	1.55-4.47	3,39E-04	2.13	1.16-3.92	1,46E-02	2.40	1.65E-05		no
5	rs1485470	C		2.71	1.56-4.71	4,03E-04	3.03	1.70-5.38	1,63E-04	2.06	0.98-4.43	5,63E-02	2.62	3.31E-05		yes
20	rs6040339	T		2.51	1.50-4.21	4,66E-04	2.93	1.70-5.04	1,02E-04			>0.05	2.64	3.88E-05		yes

Genome-wide significant signals are highlighted in grey. Chr: chromosome; SNP: single nucleotide polymorphism; OR: odds ratio; GRS: genetic risk score.

anti-TNF exposure between patients with fibrostenosis and those without fibrostenosis for more than ten years. Evidence for fibrosis was based on solely CT/MR in fourteen (18%) patients, one (1%) had stricturoplasty and 61 (80%) had surgery and pathology reports. Based on multiple logistic regression with disease location as covariate, focussing on the SNPs associated in the discovery cohort, two SNPs (rs9325636 and rs12072417) reached the 0.05 significance level (Table 2).

In meta-analysis, five of the eight associated SNPs from the discovery and validation cohort remained significantly associated, and an additional seven reached the 10^{-5} significance level (Table 2). In the latter, four signals arose from similar locations on chromosome 14, which are in LD (r^2 between 0.88-0.99). Seven of the SNPs associated with early fibrostenosis in the meta-analysis are located within known genes: *epidermal growth factor* (*EGFR*, rs4947982, c.89-1702G>A), *cadherin 4* (*CDH4,* rs4925207, c.940-2694C>T), *MIS18 binding protein* (*MISPB1,* rs35223850, p.cys714=), and an unassigned open reading frame (*LOC105370547*, rs17106208, rs17106237, rs17106213 and rs10143427).

Allocation of a genetic risk score for the development of early fibrostenosis

A genetic risk score was calculated for each individual using SNPs associated in the meta-analysis, together with those from the covariate analysis in the discovery cohort (Table 2). SNPs in LD were excluded, leaving 11 SNPs to generate the GRS. The GRS in both the discovery and validation cohort was significantly higher in the cases compared to the controls (0.29 versus -1.06, and -0,42 versus -0.93 respectively, P<0.0001, Figure 2A). To determine whether this GRS may also predict the development of fibrostenosis later than five years following diagnosis, the GRS was calculated in a group of patients from whom CT/MR and Immunochip data were available and who developed fibrostenosis between six to ten years after diagnosis (N=38). The mean GRS in this group was not different from those who developed no fibrostenosis for at least ten years (Figure 2A).

The AUC calculated from the GRS in the discovery cohort was 0.885 (Figure 2B). A cut-off value yielding 80% sensitivity and 81% specificity (log[GRS]=-0.53) was used to predict fibrostenosis in the validation cohort, resulting in an OR of 2.65 [95%CI 1.45-4.85], a Pearson Chi-square of 9.29 ($P=2.00 \times 10^{-3}$), and a positive predictive value of 54% to predict early fibrostenosis (negative predictive value: 69%).

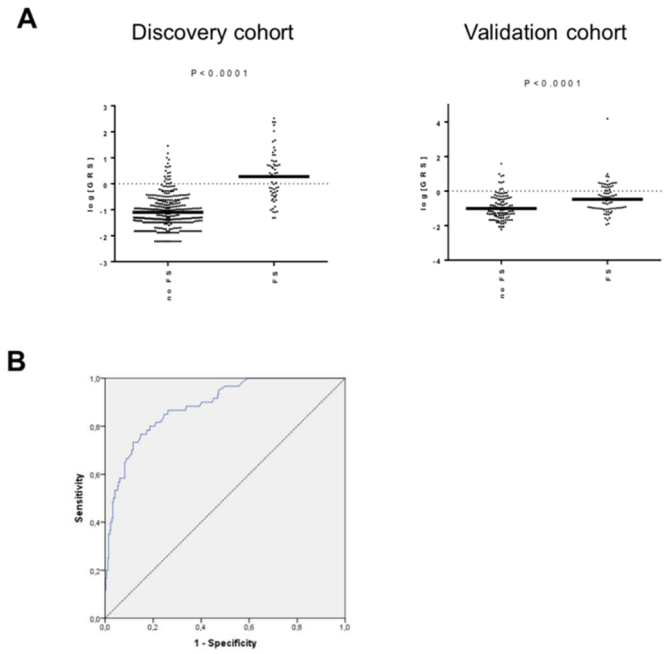

Figure 2. A genetic risk score discriminating Crohn's disease patients who developed fibrostenosis within five years versus those without fibrostenotic complications for at least ten years. Based on 11 associated SNPs, a genetic risk score (GRS) was calculated, and (A) was plotted in the controls (no FS) and cases (FS 0-5 yrs) form the discovery and the validation cohort. Additionally, a group of patients developing fibrostenosis within six to ten years (FS 6-10 yrs) following diagnosis was included. ANOVA with Sidak's multiple comparison test, or t test with Welch's correction, bars represent the mean value. (B) ROC curve generated with the GRS in the discovery cohort (AUC: 0.885). FS: fibrostenosis; yrs: years.

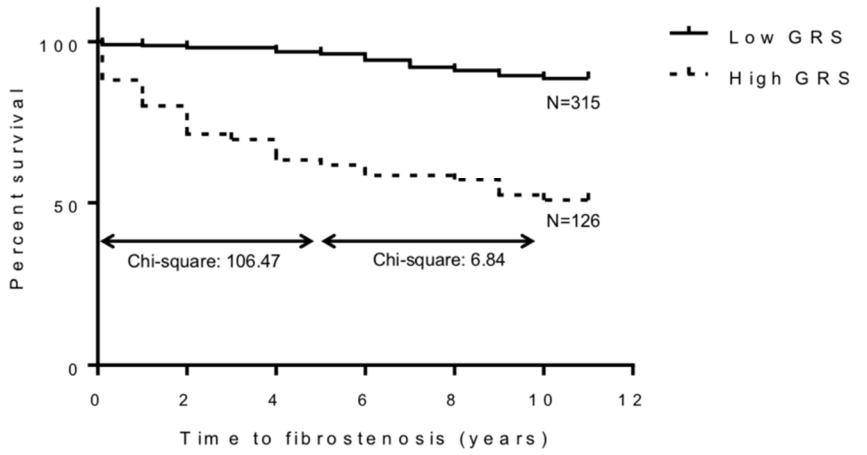

Figure 3. Influence of the genetic risk score on time to development of fibrosis. Kaplan-Meier survival plot representing the percent survival of developing fibrostenosis over time in function of a low and high genetic risk score (GRS) calculated from a cut-off value (log[GRS]=-0.53) in the discovery cohort. Chi-squares values are shown, calculated using Log Rank tests (Mantel-Cox) for the group developing fibrostenosis between zero-five years after CD diagnosis, and between six-ten years after CD diagnosis, using the same control group.

Association of the genetic risk score with the time to development of fibrostenosis

The GRS was not significantly different between patients who developed stenosis already at diagnosis and those who developed fibrostenosis within five years, nor in the discovery nor the validation cohort (mean difference 0.34±0.27 years and 0.12±0.20 years, respectively). However, when patients were assigned to a low or high GRS group, based on the cut-off value from the AUC analysis, the time to development of fibrostenosis was significantly different between patients with a high and low GRS in Kaplan-Meier analysis (Log Rank Chi-square: 90,92, P<0.0001, Figure 3). The frequency of developing fibrostenosis after two years was 29% in the high GRS group compared with 2% in the low GRS group, and amounts to 38% versus 4% after five years, and 49% versus 11% after ten years. The Chi-square was 106,47 for developing fibrostenosis within five years (P<0.0001), whereas this was 6,84 (P<0.009) for the group of patients developing fibrostenosis between six to ten years.

DISCUSSION

In this carefully phenotyped genetic association study using the Illumina Immunochip genotyping platform, CD patients with early fibrostenosis, i.e. occurring within five years after diagnosis of CD, were compared with CD patients with a longstanding disease without any indication of fibrostenotic or fistulizing disease. Genetic association was tested in a multi-center discovery and a validation cohort. Meta-analysis identified nine independent SNPs associated with the early fibrostenotic phenotype, providing evidence for an important genetic contribution to the rapid onset of fibrostenosis in CD. In addition, we provide a cut-off value of a genetic risk score (GRS), which was able to predict early stricturing disease in over half of the patients in a validation cohort, with a negative predictive value of 69%. More interestingly, the time to development of fibrostenosis was significantly linked with the GRS, i.e. the proportion of patients who developed fibrostenosis after five years was 38% in patients with a high GRS compared with 4% in the low GRS group. Although this may not be sensitive enough to adapt clinical management on individual basis, it may serve to identify patients prone for early complicated disease at diagnosis and may be used as a stratification tool for inclusion of patients in clinical trials for anti-fibrotic trials.

The GRS was also assessed in patients developing fibrostenosis between six to ten years following diagnosis, which was similar as in CD patients with longstanding disease without fibrostenotic disease for more than ten years. This contributes to our initial hypothesis that the genetic risk for fibrostenosis may be stronger in patients who develop stricturing disease early on in the disease process.

Of the nine associated SNPs in the meta-analysis, three are located within known genes. Rs35223850 is a synonymous variant in cysteine at position 714 of *MIS18BP1*, encoding a component of the Mis18 complex (Mis18α/Mis18β/Mis18BP1) which is involved in centromere maintenance during the cell cycle. It allows for the correct deposition of the centromere protein A nucleosome, required for proper chromosome segregation during normal mitosis.[17,18] Interestingly, the expression of *Mis18bp1* was significantly upregulated in models for renal fibrosis.[19] Two other interesting risk genes identified in this study include *CDH4* (rs4925207, intronic variant) and *EGFR* (rs4947982, intronic variant). Cadherin 4 is a calcium-dependent, transmembrane, cell-cell adhesion glycoprotein which is mainly involved in neuronal development. Members of the cadherin family play an important role in epithelial-to-mesenchymal transition.[20] In a rat model of cardiac fibrosis, *Cdh4* expression was significantly increased upon intermittent hypoxia exposure.[21] Epidermal growth factor receptor is a transmembrane receptor tyrosine kinase that has long been characterized as an oncogene, however, it plays an important role in mechanosensing of extracellular matrix resistance since inhibition of EGFR

signalling eliminates fibronectin strain-sensing capacities of mesenchymal stem cells.[22] Therefore, this gene should be considered as a good functional candidate gene in the pathophysiology of intestinal fibrosis.

An important strength of this study is the robust phenotyping that was performed. Based on CT/MR enterography, only patients with definite fibrostenosing CD within five years of diagnosis were selected. Moreover, over 80% of cases were additionally confirmed on surgery or histopathology, endorsing the carefully selected nature of the population. Also, results from this association study were replicated in an independent cohort. Further validation of the results in other cohorts will now be necessary.

Similar to other genotype-phenotype studies focusing on complicated CD, confounding risk factors such as disease location and disease severity is a concern. In this study, the potential bias introduced by disease location was addressed by selecting only patients with ileal disease involvement (Montreal L1 or L3), and including a second correction for L1 versus L3 in the association analyses. However, a limitation of the study is the absence of inclusion of stringent measures to assess disease severity in the case and controls groups. The use of anti-TNF did not differ between cases and controls in the discovery cohort, providing an indication that disease severity may be similar in both groups. This was however not the case in the validation cohort, in which the proportion of patients using anti-TNF was significantly higher in the control group.

In conclusion, based on a genotype-phenotype association study comparing a carefully selected population of patients with early fibrostenotic CD with CD patients with longstanding disease and lack of fibrostenotic complications, several genetic risk factors were identified. Although functional studies will be required to assess the exact role of these variants in the pathophysiology of intestinal fibrosis, these data suggest an important genetic contribution to early fibrostenotic disease, and may help in the early identification of patients prone for complicated disease.

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III. Identifying novel serum biomarkers for fibrostenotic Crohn's disease: an exploratory pilot study

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Grant support:

TH, DL are paid by grants from the Research Foundation – Flanders (11S7416N and 1298213N).

Abbreviations:

CD	Crohn's disease
CRP	C-reactive protein
CT	Computed Tomography
ECM	Extracellular Matrix
GDF15	Growth/differentiation factor 15
MMP	Matrix metalloproteinase
MRI	Magnetic Resonance Imaging
TGFβ	Transforming Growth factor β
TIMP	Tissue inhibitor of Matrix metalloproteinase

Keywords: biomarker, fibrosis, glycomics, matrix metalloproteinases, inflammatory bowel disease

Author contributions:

TH, MDV, DL, XV, PH, SB: study concept and design

TH, SB, XV: acquisition of data and analysis

TH, MV, PH, DL: writing of the manuscript

All authors: critical revision of the manuscript

Status

Manuscript in preparation for submission

ABSTRACT

Background:

Fibrostenosis is a frequently occurring complication of longstanding Crohn's disease (CD). However, accurate diagnosis of fibrostenosis, and differentiation between inflammatory and fibrotic stenoses are difficult, compromising clinical management and construction of clinical trials. The aim of this exploratory study was to identify potential serological biomarkers identifying the presence of fibrostenosis.

Methods:

In this single-centre, retrospective, case-control study, fibrostenotic CD was defined by the presence of ileal disease and bowel wall thickening with luminal narrowing and prestenotic dilatation on computed tomography (CT) or magnetic resonance (MR) enterography. The control cohort consisted of age- and gender-matched CD patients with ileal and inflammatory disease for a minimum of 10 years without fibrostenotic or penetrating complications. Active inflammation was defined as CRP levels (> 5 mg/dl) and/or the presence of active luminal disease on endoscopic or radiological assessment. MMPs, TIMPs and glycomics were quantified in serum samples.

Results:

A total of 1,221 CT or MRI scans of 1,085 CD patients were screened, resulting in a diagnosis of fibrostenosis in 223 (20.6%) of which 32 patients were randomly selected. Thirty-two age- and sex-matched patients with inflammatory CD and eight healthy controls were included. MMP-2, MMP-3 and TIMP-3 were differentially expressed between fibrostenotic and inflammatory CD in patients with active inflammation. Combining these markers resulted in an AUC of 0.855, while a cut-off lower than 2,328 resulted in a sensitivity of 87.5%, a specificity of 81.8% of predicting fibrostenosis with a positive predictive value of 82.35% and a negative predictive value of 86.67%. In a subcohort of serum samples collected prior to the development of fibrostenosis, MMP-10 levels were lower than in patients with uncomplicated disease.

Conclusions:

In this exploratory study, MMP-2, MMP-3 and TIMP-3 were identified as potential biomarkers for fibrostenotic CD, while MMP-10 might be able to predict occurrence of fibrotic complications prior to the onset of fibrostenosis. Confirmation in validation cohorts is necessary.

INTRODUCTION

Crohn's disease (CD) is a chronic disorder of the gastrointestinal tract characterized by episodes of relapsing-remitting inflammation mainly affecting the ileum and colon. Over time, these recurrent episodes of transmural inflammation can cause deposition of extracellular matrix (ECM) in the (sub)mucosa and result in fibrostenosis, characterized by the development of luminal stenosis and organ failure, affecting about one third of CD patients.[1] Currently, surgery is the only therapeutic option with an associated loss of viable intestinal tissue and high recurrence rates of up to 70-80%.[2,3] Although several anti-fibrotic agents have been tested in a preclinical setting, they have difficulties progressing to the clinical trial stage. [4] Aside from the longevity of such trials, one of the main problems associated with constructing clinical studies in fibrostenotic CD is the lack of an accurate fibrosis marker allowing for the selection of patients at risk and use as a surrogate marker to assess therapeutic effects. Additionally, such a fibrosis marker could be useful in clinical practice to differentiate between inflammatory and purely fibrotic strictures, the former being amendable by anti-inflammatory therapy while the latter are only suitable for surgical intervention.

Cross-sectional imaging such as computed tomography (CT) or magnetic resonance imaging (MRI) can discriminate between inflammatory and fibrotic strictures to some extent, but provide no quantifiable way of following fibrosis over time.[5] Although potential biomarkers for fibrosis in other organs have been identified, none of these markers were linked with fibrostenotic CD. [6,7] Most of these markers are structural or regulatory proteins that play a role in fibrogenesis, such as transforming growth factor beta (TGFβ) and other growth factors from the TGFβ superfamily such as growth/differentiation factor 15 (GDF15). Both have been suggested as biomarkers in systemic sclerosis, cardiovascular and liver fibrosis. [8-10] Similarly, matrix metalloproteinases (MMP), zinc and calcium-dependent endopeptidases involved in degradation of the ECM have, together with their inhibitors (TIMPs), shown diagnostic value in pulmonary and liver fibrosis, making the interesting candidates to act as biomarker in intestinal fibrosis. [11-13]

Another entirely different approach is to look at differential glycosylation patterns of serum proteins which has provided promising biomarkers in liver fibrosis. Glycosylation represents a post-translation modification in which glycan are attached to protein, lipids or other organic molecules. Approximately 50% of serum proteins have undergone glycosylation and many of these are produced and secreted in gastrointestinal tissues. N-glycolysation, the attachment of a glycan to a nitrogen atom (provided by an amide nitrogen or Asparagine amino acid), is the most prevalent form of serum protein

glycolysation and alterations in the total serum N-glycolysation pattern have been shown to correlate well with liver fibrosis. [14-16]

The aim of this exploratory pilot study was to investigate performance of known fibrotic markers in a well-phenotyped population of fibrostenotic CD, identified based on CT/MRI imaging.

MATERIALS AND METHODS

Study design and patient selection

Crohn's disease was diagnosed based on clinical, endoscopic and histological criteria. In this single-centre, retrospective case-control study, performed at the University Hospital of Ghent, CT and MRI enterography scans from CD patients with ileal or ileocolic CD (Montreal L1 or L3), obtained between 2002 and 2016, were examined for the signs of fibrostenotic disease, defined as the presence of bowel thickening with luminal narrowing and prestenotic dilatation. The control cohort consisted of CD patients (Montreal L1 or L3) with an inflammatory disease course, without clinical, radiographic or endoscopic arguments for fibrostenotic or fistulising disease for at least ten years of follow-up. Only patients with available serum samples were included. Patients with evidence for extra-intestinal fibrosis were excluded, specifically the presence of idiopathic pulmonary fibrosis, liver fibrosis, congestive heart failure, scleroderma or renal fibrosis. For the sub-analysis, patients were divided according to presence of active disease at the time of the blood sampling, based on CRP levels (> 5 mg/dl) and/or the presence of active luminal disease on endoscopic or radiological assessment. This study was approved by the ethical committee of UZ Ghent (EC number 2016/0761). All patients gave written informed consent prior to inclusion.

Serum measurement of biomarkers

Protein levels of MMPs and TIMPs in serum samples were determined using the Luminex panel, according to the manufacturer's guidelines (Bio-Rad, Temse, Belgium). Serum levels of GDF15 were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D, Oxon, UK) per manufacturer's guidelines.

Serum glycomics

Materials

Ammonium Acetate (NH_4Ac), Sodium Dodecyl sulphate (SDS), citric acid, Sodium cyanoboro hydride 95% ($NaBH_3CN$), Dimethyl sulfoxide (DMSO) and APTS (8-Aminopyrene-1,3,6-trisulfonic acid) were purchased by Sigma Aldrich. Nonidet-40 (NP-40) was purchased by Fluka Biochemika. The 96-well PCR Plate, non-skirted were purchased by Thermo Scientific and the MicroAmp Optical 96-Well Reaction

Plate by Applied Biosystems. The enzymes Arthrobacter ureafaciens α(2 ->3,6,8,9)-silaidase (200 mU/µL) and PNGase F (600 U/µL NEB) were provided by the Molecular Glycobiology Unit of the Department for Molecular Biomedical Research, Flanders Institute for Biotechnology (VIB), Technologiepark (Ghent, Belgium).

Sample protein N-glycome sample processing

The N-glycans present on the proteins in 3 µl of the serum samples were released after protein binding to a 96-well PCR plate, derivatized with 8-aminopyrene-1,3,6-trisulfonic acid (APTS), desialylated and analyzed by DSA-FACE (DNA sequencer-assisted flurophore-assisted carbohydrate electrophoresis, Applied Biosystems). The optimized protocol for glycan release and labeling using a PCR thermocycler is as follows: 2 µl of 10 mM NH_4Ac buffer, pH 5 containing 3.5% SDS were added to 3 µL of serum in 96 well PCR plate. Each sample was mixed by up and down pipetting without creating any bubbles. The tightly closed tubes were centrifuged for 1min at 335g and heated at 95 °C for 5 min in a standard PCR thermocycler with heated lid. After cooling, 5 µl of 10 mM NH_4Ac, pH 5, containing 2% NP-40 solution and 2.5 IUBMB milliunits of peptide N-glycosidase F (PNGase F, Glyko) were added. The tubes were mixed, centrifuged and incubated in the PCR thermocycler for 10 min at 37°C with lid closed at 40°C. Subsequently, 5 µl of 100 mM NH4Ac, pH5 with 40 milliunits of α(2 3,6,8,9)-silaidase were added, followed by up and down mixing. The tubes were tightly closed, centrifuged and incubated in the PCR thermocycler for 60 min at 37°C with lid closed at 40°C. Following the incubation, samples were evaporated to dryness, for 20 min at 80°C with tubes open. The evaporation was complete, after which 5 µl of the labeling solution (1:1 solution of 20 mM APTS in 1.2 M citric acid and $NaBH_3CN$ in DMSO) were added to the bottom of the tubes. The tightly closed tubes were then vortexed, centrifuged and heated at 50 °C for 2 h (the elevated temperature ensures fast reaction kinetics). Water (200 µl) was then added to each tube to quench the reaction and dilute the label 1:125 and 1:625.

Data management

20 µL of the resulting solutions were used for analysis by DSA-FACE. Data analysis was performed using the GeneMapper software v3.7 (Applied Biosystems). We quantified the heights of the 13 peaks that were detectable in all samples to obtain a numerical description of the profiles, and analyzed these data. Absolute raw data of the peak heights were normalized (in %) to their abundance to the total peak height intensity.

Biomarker selection

Biomarkers were selected based on their differential expression between patients with stenotic and inflammatory CD. To increase the discriminative ability several biomarkers interested were combined using the following formula: (MMP-2*MMP-3)/TIMP-3.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad, California, USA) and SPSS Statistics version 22 (IBM Corp, Chicago, USA). Student's *t* tests were used to compare differences between two groups for normally distributed data. In cases of non-normal distributions, data were log-transformed or were analyzed using Mann-Whitney U tests. To compare continuous variables between more than two groups, the Kruskal-Wallis test was used. Receiver-operator curves (ROC) were generated to determine the diagnostic accuracy of the tests. Kaplan-Meier survival curves were compared to evaluate the influence of biomarkers on time to development of fibrosis.

RESULTS

Patient population

A total of 1,221 CT or MRI scans of 1,085 CD patients with ileal or ileocolonic disease was reviewed. Based on these data, 223 patients (20.6 %) were classified as fibrostenotic disease, defined as the presence of bowel wall thickening with luminal narrowing and prestenotic dilatation. Out of this population, 32 patients were selected randomly for inclusion in this study. An additional 16 patients were included who had serum samples available collected prior to any evidence of fibrostenotic disease. Thirty-two age- and sex-matched controls were randomly selected from a cohort of patients with an inflammatory disease course (defined as having no evidence for stricturing or penetrating disease for at least ten years after CD diagnosis). Additionally, 8 age- and sex-matched healthy controls were included.

Serum biomarkers for fibrostenotic CD

Mean GDF15 serum levels were significantly higher in serum samples of CD patients compared with healthy controls (P<.001), but were unable to discriminate between patients with fibrostenotic or inflammatory CD (Figure 1A). Serum TIMP-3 levels were elevated in the patients with fibrostenotic CD compared with patients with inflammatory CD (P<.01) or healthy controls (P=.08). None of the other measured TIMPs differed significantly between groups (Figure 1B).

MMP-2, -3 and -9 levels were significantly lower in serum samples of CD patients compared with healthy controls (P<.05), while MMP-8 levels were higher (P<.05). None of these analytes could discriminate serum samples of fibrostenotic or inflammatory CD patients (Figure 2). No difference was observed in MMP-11 and -12 levels between healthy controls and CD patients, while MMP-1 levels were significantly lower in patients with fibrostenotic CD compared to healthy controls, but not in the inflammatory CD group (P<.05).

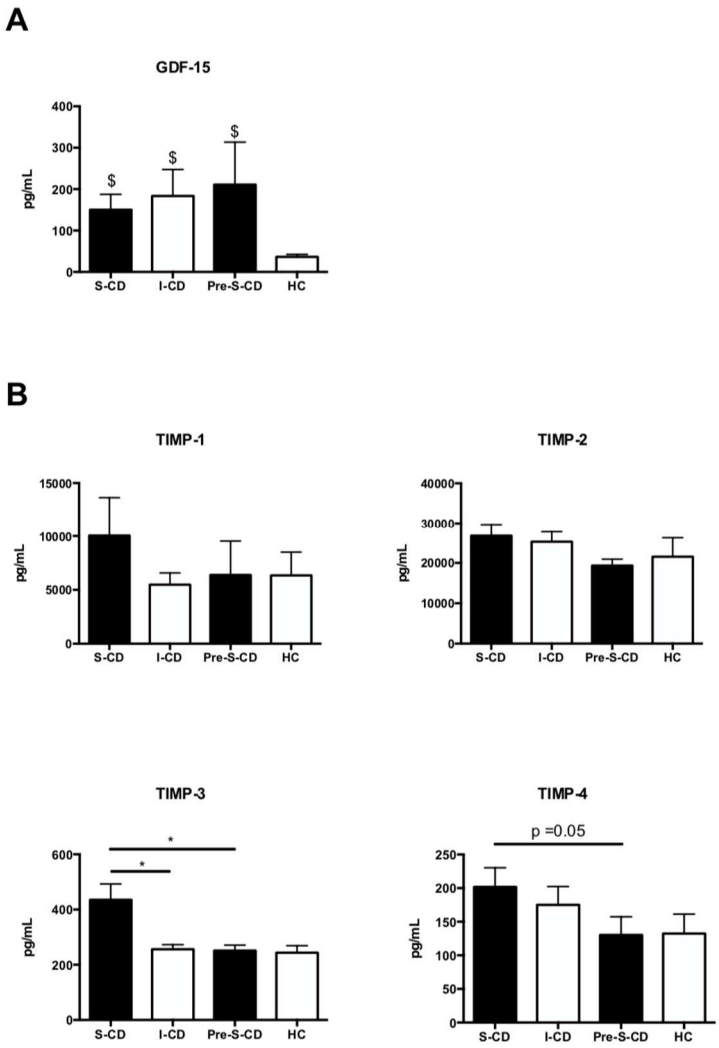

Figure 1 - TIMP-3 levels are elevated in patients with fibrostenotic Crohn's disease

(A) Serum GDF-15 levels determined by ELISA assay (B) Serum TIMP levels measured by Luminex magnetic bead technology. $ *P* < .05 compared to healthy controls, * *P* < .05; ** *P* < .01. S-CD: fibrostenotic Crohn's disease (N=32), I-CD: inflammatory Crohn's disease (N=32), pre-S-CD: serum samples of fibrostenotic Crohn's disease patients collected before occurrence of fibrotic complications (N=16), HC: healthy controls (N=8); GDF: growth differentiation factor, TIMP: tissue inhibitors of matrix metalloproteinases

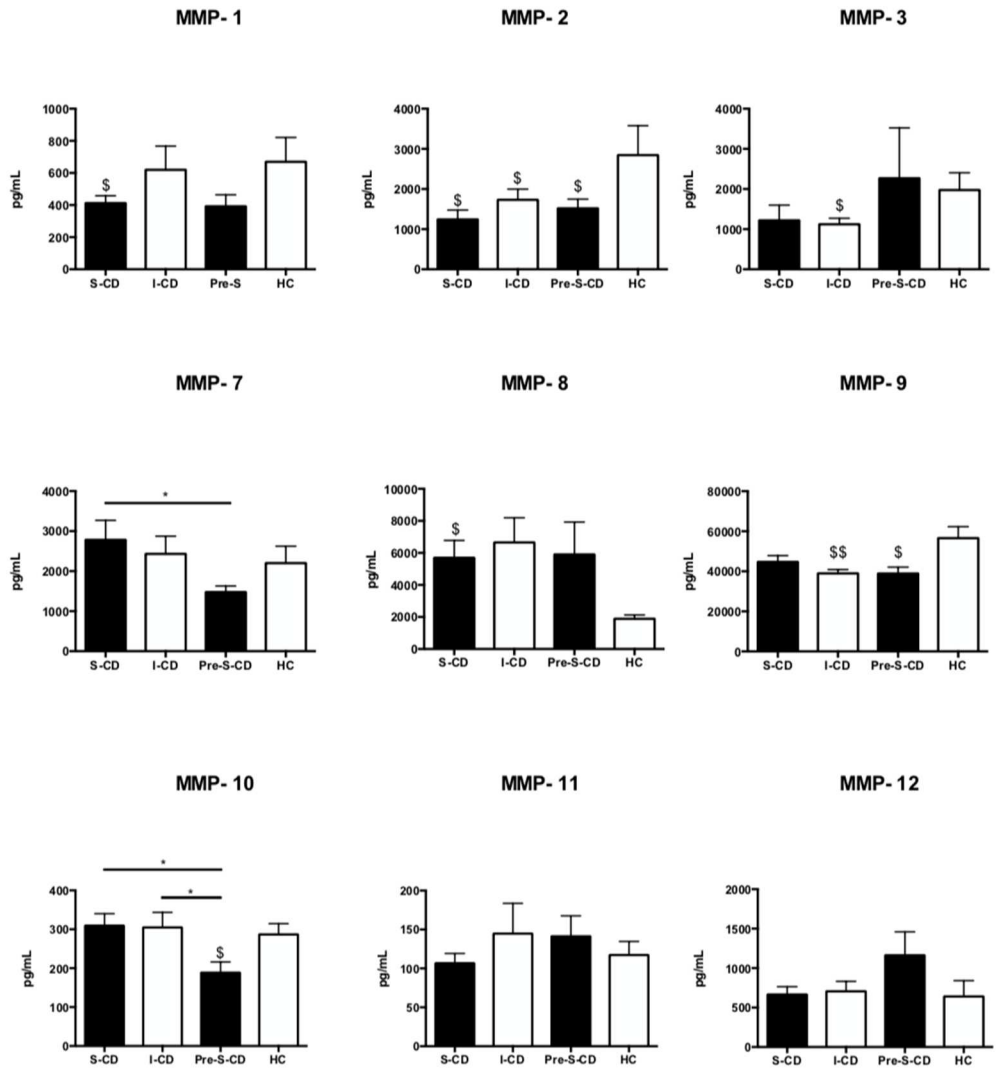

Figure 2 – Serum MMPs are not differentially expressed between fibrostenotic and inflammatory Crohn's disease

Serum MMP levels measured by Luminex magnetic bead technology. $ *P* < .05 compared to healthy controls, * *P* < .05; ** *P* < .01. S-CD: fibrostenotic Crohn's disease (N=32), I-CD: inflammatory Crohn's disease (N=32), pre-S-CD: serum samples of fibrostenotic Crohn's disease patients collected before occurrence of fibrotic complications (N=16), HC: healthy controls (N=8); MMP: matrix metalloproteinases

Sixteen patients had serum samples available prior to development of fibrostenotic disease, which could potentially identify predictive markers. TIMP-3 and TIMP-4 serum levels were significantly higher (*P*<.05) in patients with already established fibrostenotic disease compared with those who had not yet developed the complication, suggesting that these markers are specific for established fibrostenosis. However, they did not allow to discriminate pre-stenotic samples from those collected from patients with a purely inflammatory disease course (Figure 1B). Similarly, MMP-7 and -10 levels were significantly elevated in serum samples of fibrostenotic CD patients compared with patients who

have not yet developed fibrotic complications (*P*<.05) (Figure 2). Interestingly, MMP-10 levels were significantly higher in samples collected from the inflammatory CD group compared with the pre-stenotic samples (Figure 2) (P<.05). In ROC analysis, MMP-10 levels had a AUC of 0.716 for predicting fibrostenotic disease (Figure 5A) (95% CI 0.564 – 0.867; *P* = .016).

Influence of the presence of inflammation

The presence of inflammation can confound serum levels of MMPs, TIMPs and GDF-15. Patients were considered to have significant inflammation when CRP levels were higher than 5 mg/dL or if they had evidence for active luminal disease on endoscopy and/or radiology. Interestingly, in patients with evidence for inflammation, TIMP-3 levels were significantly higher in serum samples of patients with stenosis compared to patients with inflammatory disease (*P*<.01) (Figure 3), while levels of MMP-2 and -3 were lower in the stenosis group (*P*<.05) (Figure 4). ROC curves for MMP-2, MMP-3 and TIMP-3 show an AUC of respectively 0.805 (95% CI 0.655 – 0.955; P = .003), 0.768 (95% CI 0.551-0.930; P = .02) and 0.740 (95% CI 0.595-0.940; P = .01) (Figure 5B). Combining the three markers resulted in an AUROC of 0.855 (0.855; 95% CI 0.718 – 0.993; *P* = .001). Based on a cut-off value of 2,328, a sensitivity for predicting fibrostenotic disease of 87.5% and a specificity of 81.8% is achieved. The positive predictive value associated with this cut-off is 82.35% with a negative predictive value of 86.67%.

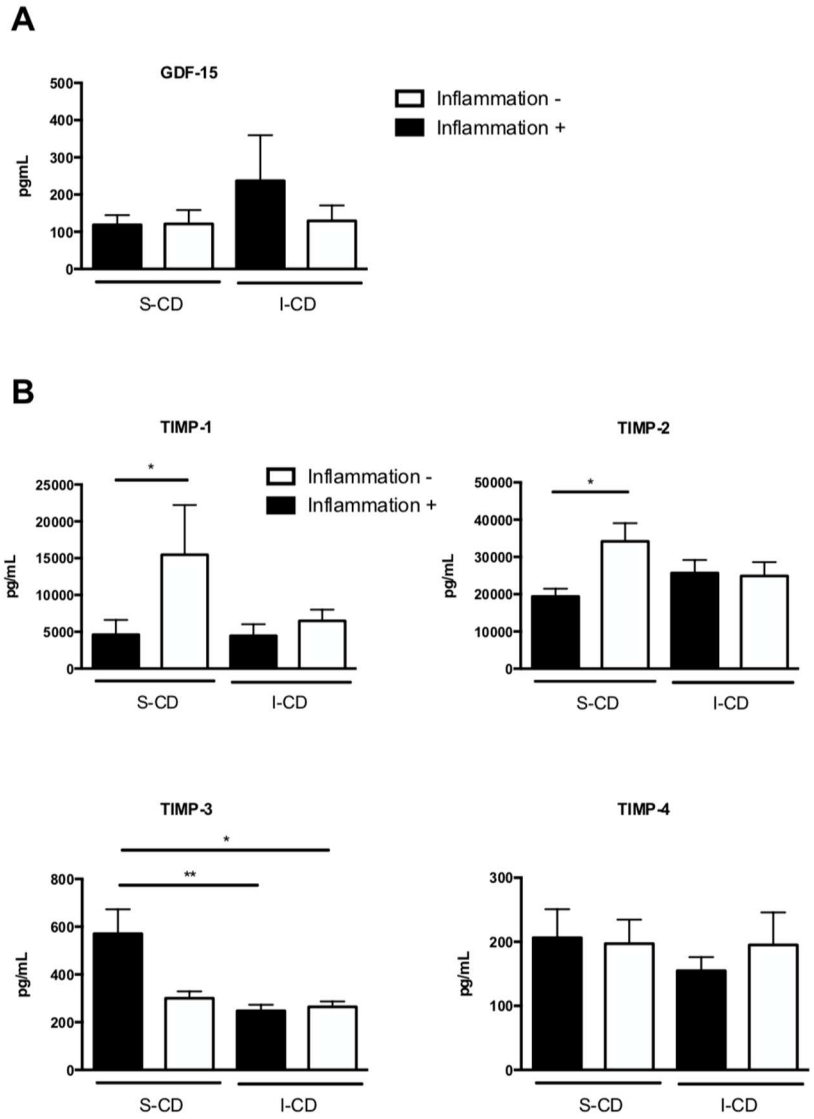

Figure 3 – Serum TIMP-3 levels discriminate between fibrostenotic and inflammatory Crohn's disease in patients with active inflammation

(A) Serum GDF-15 levels determined by ELISA assay (B) Serum TIMP levels measured by Luminex magnetic bead technology. Presence of inflammation defined as CRP levels > 5 mg/dL and/or the presence of active luminal disease on endoscopic or radiological assessment. * $P < .05$; ** $P < .01$. S-CD: fibrostenotic Crohn's disease (N=32), I-CD: inflammatory Crohn's disease (N=32), GDF: growth differentiation factor, TIMP: tissue inhibitors of matrix metalloproteinases

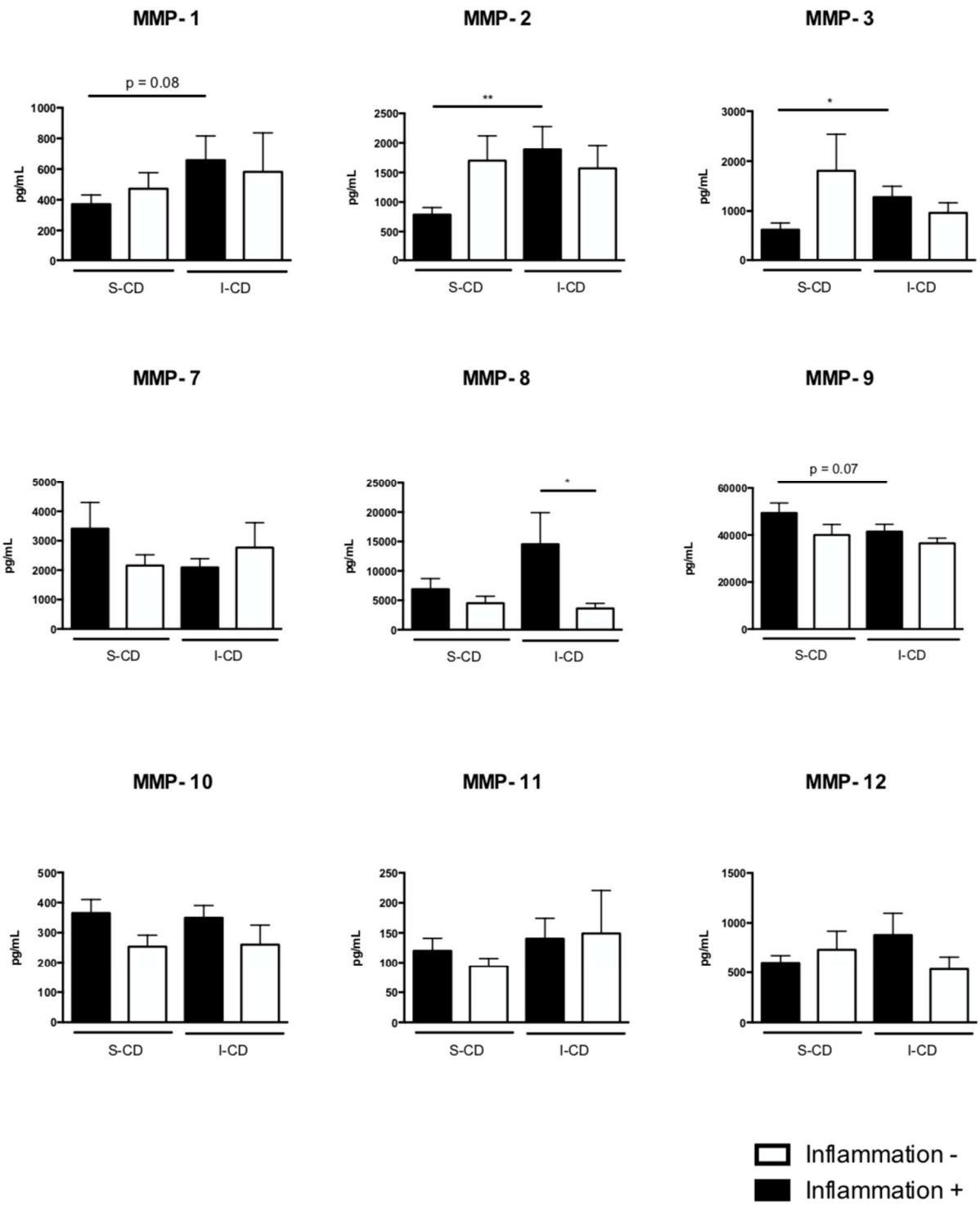

Figure 4 – Serum MMP-2 and -3 levels discriminate between fibrostenotic and inflammatory Crohn's disease in patients with active inflammation

Serum MMP levels measured by Luminex magnetic bead technology. Presence of inflammation defined as CRP levels > 5 mg/dL and/or the presence of active luminal disease on endoscopic or

radiological assessment. * $P < .05$; ** $P < .01$. S-CD: fibrostenotic Crohn's disease (N=32), I-CD: inflammatory Crohn's disease (N=32); MMP: matrix metalloproteinases

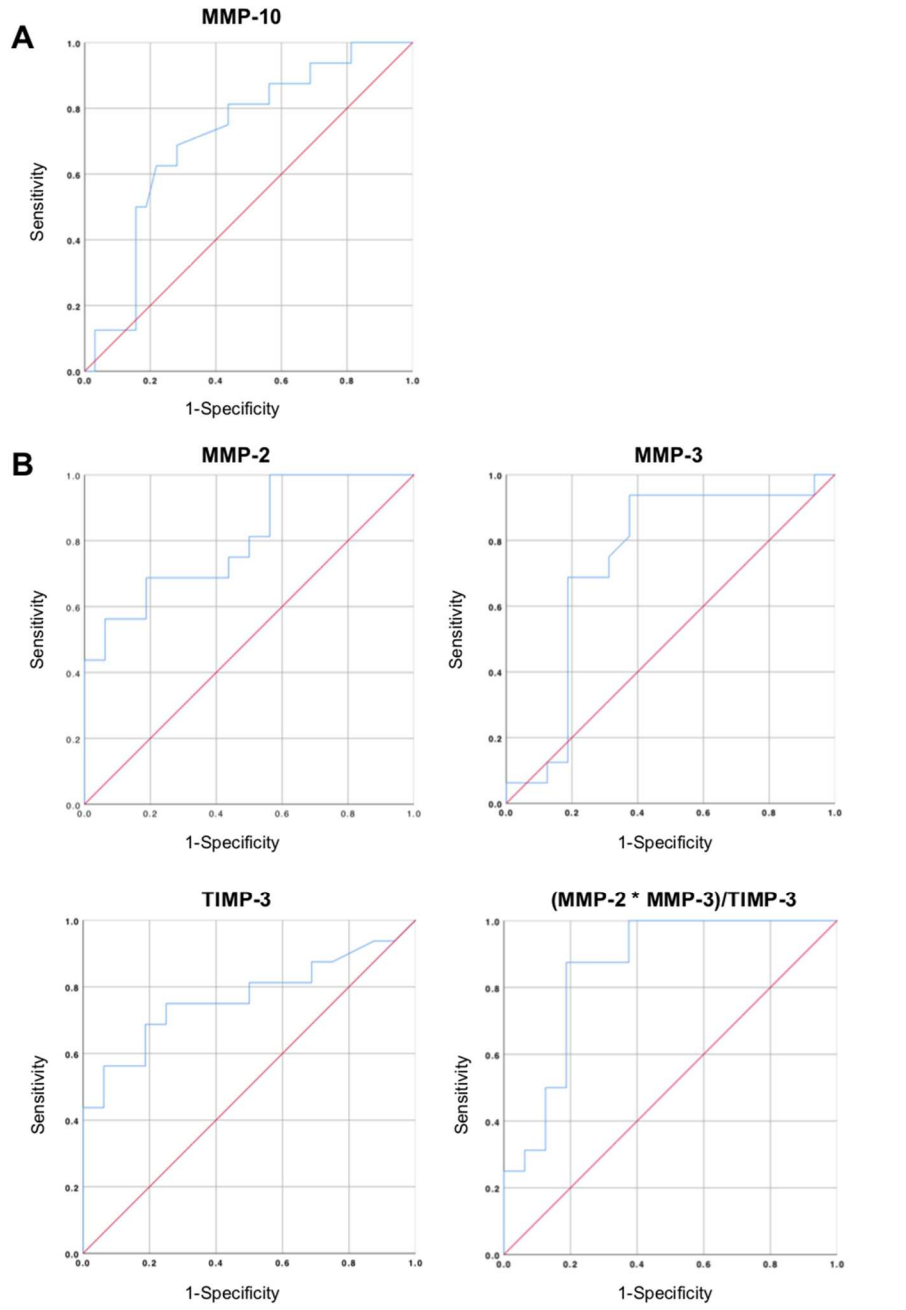

Figure 5 – ROC curves for potential biomarkers of fibrostenotic Crohn's disease

(A) ROC curve showing diagnostic accuracy of serum MMP-10 in predicting fibrostenotic complications prior to occurrence of events (N=16). AUC = 0.714; 95% CI 0.564 – 0.867; *P* = .016. **(B)** ROC curves showing diagnostic accuracy of serum MMP-2, MMP-3 and TIMP-3 levels for diagnosis of fibrostenotic complications in patients with evidence of active inflammation (defined as CRP levels > 5 mg/dL and/or the presence of active luminal disease on endoscopic or radiological assessment). AUC MMP-2 = 0.805; 95% CI 0.655 – 0.955; P = .003; AUC MMP-3 = 0.740; 95% CI 0.551-0.930; P = .02; AUC TIMP-3 = 0.768; 95% CI 0.595-0.940; P = .01; AUC (MMP-2*MMP-3)/TIMP-3 = 0.855; 95% CI 0.718 – 0.993; *P* = .001. CRP = C-reactive protein; MMP = matrix metalloproteinase, TIMP = tissue inhibitor of MMP; ROC = receiver operator curve, AUC = area under the curve

Influence of biomarkers on the time to development of fibrosis

In the patients with fibrostenotic disease, the serum levels of MMP-2, -3 or TIMP-3 did not significantly affect the time to development of fibrosis. However, for MMP-2 there was a non-significant trend (*P*=.12) towards faster development of fibrostenosis in the patients with MMP-2 concentrations in the lowest quartile compared with patients with concentrations in the highest quartile (Figure 6A). Moreover, significantly more patients in this lowest quartile of MMP-2 concentrations already had established fibrostenosis at the time of CD diagnosis (71% vs 37.5% in the other quartile ranges; *P*<.001) (Figure 6B).

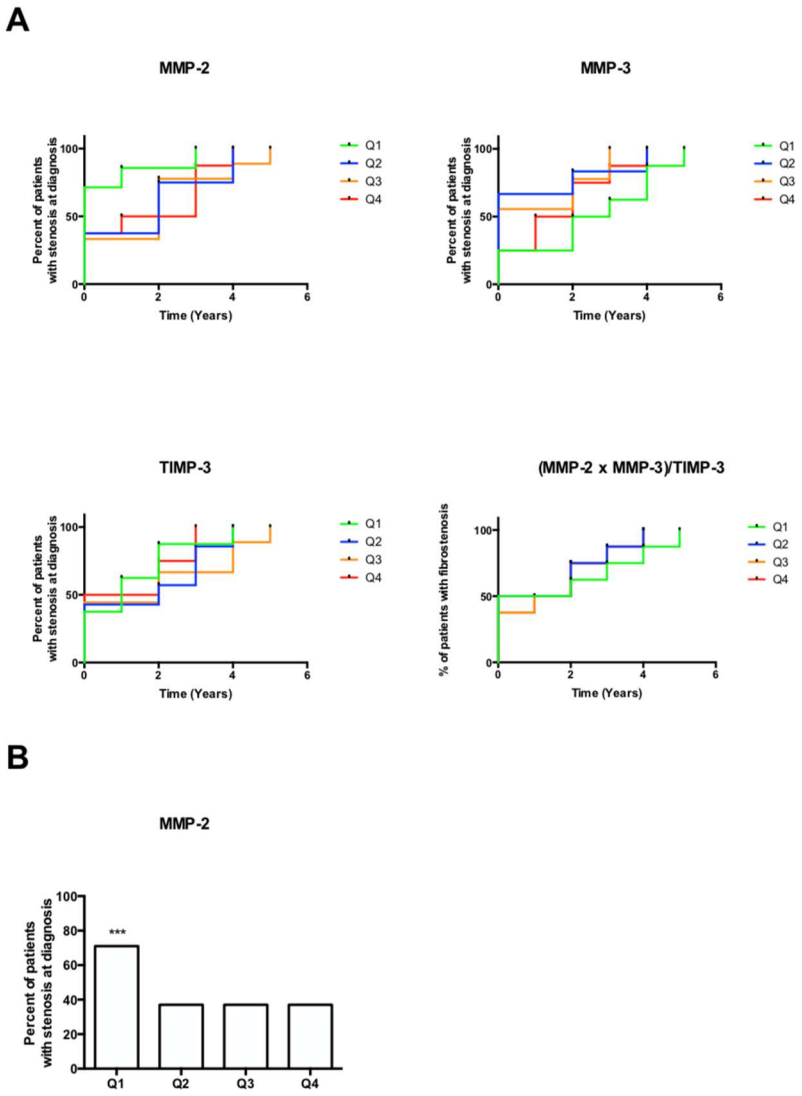

Figure 6 – Lower MMP-2 levels are associated with a trend towards faster development of fibrostenosis

(A) Kaplan-Meier survival curves representing time to development of fibrosis (in years) across different quartiles of serum levels of MMP-2, MMP-3 and TIMP-3 (N=32) **(B)** Proportion of patients with already

established fibrosis at the moment of Crohn's disease diagnosis across quartiles of MMP-2 serum levels *** P < .0001; MMP = matrix metalloproteinases, TIMP = tissue inhibitors of metalloproteinases

Serum glycomics

Additionally, N-glycolysation patterns of total serum proteins were compared between groups using a protocol specifically designed for liver fibrosis. Thirteen peaks could be quantified in the serum samples and were normalised to the total peak height intensity per patient. Peak 6 and 9 were able to discriminate between healthy subjects and patients with CD (*P*<.05). However, no differential glycomic signature could be discovered between patients with fibrostenotic or inflammatory CD (Figure 7).

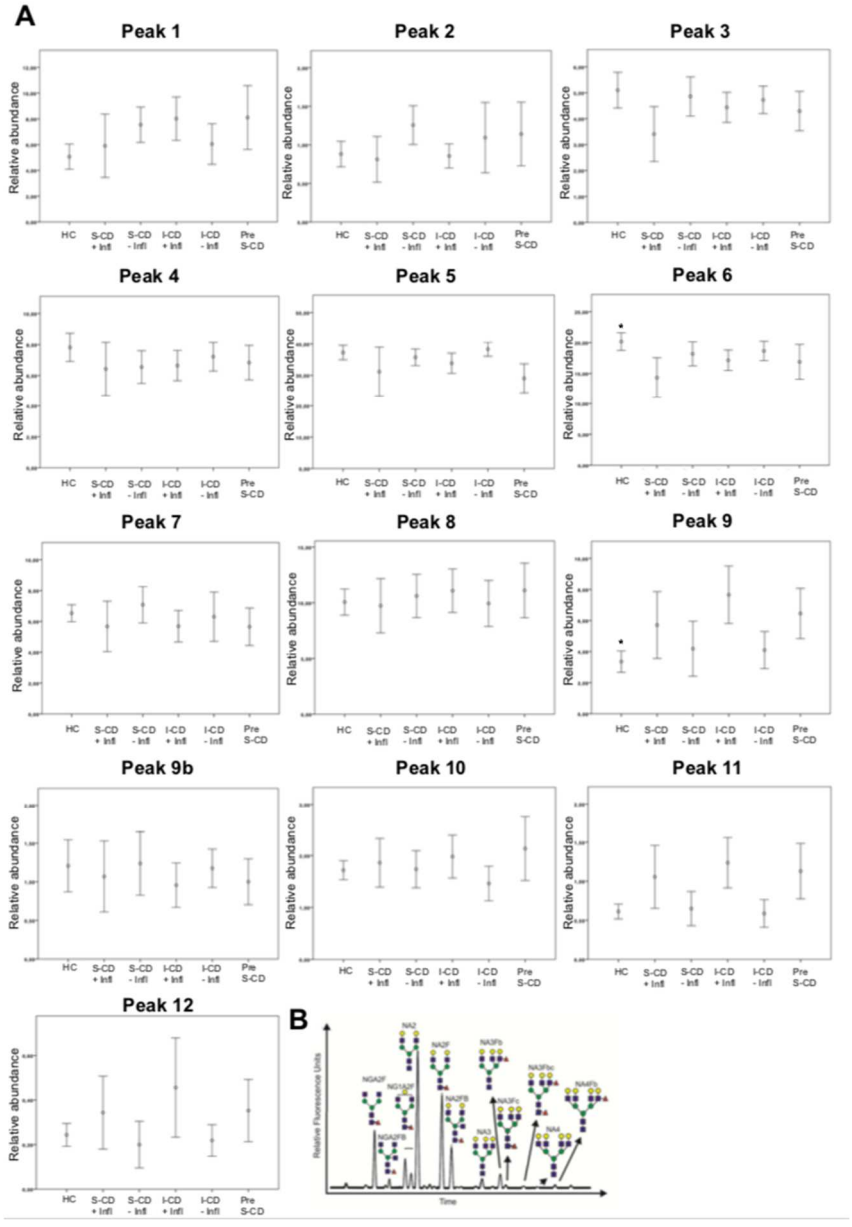

Figure 7 – N-glycosylation profiles of total serum proteins are not able to discriminate between fibrostenotic and inflammatory Crohn's disease

(A) Relative abundance of N-glycosylation patterns of total serum proteins, represented as mean ± 95 % CI. **(B)** Overview of different N-glycosylation peaks tested. * $P < .05$ N=32

DISCUSSION

In this exploratory study, the predictive value of potential biomarkers for fibrosis was evaluated in a well-phenotyped population of fibrostenotic CD. Serum levels of MMP-2, MMP-3 and TIMP-3 were found to be differentially expressed between patients with fibrostenotic and inflammatory CD. Combining these three markers resulted in a potential means to discriminate between fibrostenosis and uncomplicated CD, at least in patients with evidence of inflammation.

The lack of means to accurately discriminate between CD patients with fibrostenosis and patients who do not have this complication is an important hiatus in the current management of CD patients. Not only does it affect clinical decision making (e.g. timely referral for surgery versus medical step-up therapy), but it also hampers construction of clinical trials investigating new anti-fibrotic treatments as such trials depend on accurate inclusion of patients at risk.

Serum biomarkers are particularly attractive as they are inexpensive, easy to obtain and can be used for serial monitoring. In this preliminary study, we found 3 serum proteins to be differentially expressed between patients with fibrostenotic and inflammatory CD. MMP-2 and -3 belong to the family of matrix metalloproteinases, zinc and calcium-dependent endopeptidases that are involved in degradation of the extracellular matrix, an important component of fibrotic tissue. In CD patients who developed fibrostenosis, serum levels were lower than in patients who did not develop these complications. Conversely, serum levels of TIMP-3, belonging to a family of tissue inhibitors of MMPs that inhibit degradation by MMPs, were higher in these patients. Each of these serum proteins had a good discriminant function for predicting fibrostenotic disease in patients who had evidence of present inflammation (CRP ≥ 5 and/or presence of intestinal inflammation on endoscopy/radiology). Combining these three proteins, however, provided the best means to identify fibrostenotic disease with an AUC of 0.855. When selecting a cut-off value lower than 2,328, a positive predictive value of 82.35% and a negative predictive value of 86.67% was achieved. Why these biomarkers performed better in the presence of inflammation is unclear. However, it is in these settings when they would be most useful. Discriminating an inflammatory from a fibrotic stenosis is important for determining its management, the first being responsive to medical therapy the latter often requiring surgery. As some

degree of inflammation is usually present in these patients, determining these additional markers of fibrostenosis might help clinical decisions.

Biomarkers that can predict fibrostenosis before it occurs are even more valuable as they allow for preventive measures. In our preliminary study, 16 patients with serum samples collected before fibrostenotic complications occurred were included. Only MMP-10 levels could discriminate these patients from CD patients with a more benign, inflammatory disease course, with serum levels tending to be lower in patients who developed fibrostenosis.

Although N-glycosylation profiles of serum proteins have shown promising results in predicting liver fibrosis [14-16], this did not appear to be the case in fibrostenotic CD, at least not in this preliminary cohort. This might be due to the fact that the used panel has been optimised for use in liver fibrosis. Using an unrestricted technique looking into differentially expressed glycan-structures in general might provide better markers for fibrostenotic CD. An exploratory study in 28 patients with fibrostenotic CD undergoing surgery identified two markers (hepatic growth factor α and cartilage oligometrix matrix protein) that were differentially expressed prior and after surgery. [5] The finding that N-glycolysation profiles could discriminate between healthy subjects and CD patients is a confirmation of the findings of Trbojevic et al who found differential expression of IgG glycan profiles, a finding linked to the chronic inflammatory state present in CD patients.[17]

As this is an exploratory study, its results should be interpreted with caution and need to be confirmed in larger validation sets. The finding of MMP-10 as a potential predictive marker for the development of fibrostenotic complications is interesting but will need to be explored in prospective studies. Looking at changes in MMP-10 serum levels following surgery for fibrostenosis could be another way of further exploring the potential of this biomarker.

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IV. Oral Corticosteroids for Inducing Remission in Ulcerative Colitis

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Publication status and date: Under revision in Cochrane IBD

Abstract

Background. Oral corticosteroids are commonly used for induction of remission in ulcerative colitis (UC) with two types of agents (systemic or locally active) being available. Systemic corticosteroids such as (methyl)prednisolone especially are associated with a high risk of adverse events, while second-generation corticosteroids with limited systemic bioavailability (also called locally active oral corticosteroids) as budesonide, beclomethasone dipropionate, fluticasone propionate and prednisolone metasulfobenzoate may be associated with fewer and less severe adverse events.

Objectives. The primary objective was to assess the efficacy and safety of oral corticosteroids (both systemic and locally active) for induction of remission in active UC.

Search methods. A literature search for relevant studies (inception to 9 August 2016) was performed using MEDLINE, EMBASE and the Cochrane Library. Review articles and conference proceedings were also searched to identify additional studies.

Selection criteria. Randomized controlled trials (RCTs) that compared oral corticosteroids to placebo or an active comparator in patients presenting with active UC were included. There were no exclusions based on patient age or the type, dose, duration or formulation of the oral corticosteroid therapy.

Data collection and analysis. Two authors (TH and WV) independently screened studies for inclusion, extracted data and assessed the methodological quality of included studies using the Cochrane risk of bias tool. The overall quality of the evidence supporting each outcome was assessed using the GRADE criteria. The primary outcome measure was the number of patients achieving remission as defined by the individual studies. Secondary outcomes included: clinical, endoscopic and histological improvement; change in Disease Activity Index (DAI) (clinical symptoms and endoscopic appearance of mucosa); treatment failure requiring additional treatment; surgery (colectomy); occurrence of adverse events; and withdrawal due to adverse events. The relative risk (RR) and 95% confidence intervals (95% CI) were calculated for each outcome. Data were analysed on an intention-to-treat basis.

Main results .Twenty-three RCTs (n = 3338 patients) were identified and included in the review. Of these, 7 RCT's including 1657 patients compared oral corticosteroids to placebo. All of the included studies were rated as low risk of bias. Compared to placebo, use of oral corticosteroids for the induction of remission of active UC was associated with significantly higher rates of clinical remission (7 studies; 1657 patients; RR 2.10, 95% confidence interval (CI) 1.41 to 3.14; P < 0.001; I^2=59%, GRADE = low) and clinical response (5 studies; 1163 patients; RR 1.25, 95% CI 1.03-1.51, P = 0.02, I^2=0%,

GRADE = moderate). There was a significant difference in endoscopic (5 trials; 1013 patients; RR 2.01, 95% CI 1.25 to 3.22; I^2 = 45%; P = 0.004; GRADE = very low) and histological remission rates (4 trials, 1391 patients, RR 1.51 95% CI 1.12-2.04, I^2 = 19%; P = 0.007, GRADE = low) between patients with active UC treated with corticosteroids and placebo. The Ulcerative Colitis Change in Disease Activity Index (UCDAI; 2 trials; 155 patients; MD 0.88, 95% CI 0.21 to 1.54; I^2 = 11%; P = 0.01) did was significantly better in the corticosteroid treated patients compared to placebo. There was a trend towards higher adverse events in the corticosteroid treated groups but this was not significantly different (6 studies, 1731 patients, RR 1.09, 95% CI 0.87 to 1.37, I^2= 56%; P=0.46, GRADE = moderate). Withdrawal due to adverse events did not differ between the placebo group and the corticosteroid treated group (6 trials, 1641 patients, RR 1.00, 95% CI 0.74 to 1.34, I^2=26%, P = 0.98).

Four studies, all rated low risk of bias, compared systemic corticosteroids to locally active agents (n = 735) and found no difference in clinical remission rates, although there was a statistical trend towards patients treated with locally active corticosteroids being less likely to achieve endoscopic remission (4 trials, n = 735, RR 0.78 95% CI 0.60-1.02; P=0.07, I^2=52%, GRADE = low). The risk of adverse events, however, was lower in patients treated with locally active corticosteroids (3 trials, n = 668 patients, RR 0.67 95% CI 0.54-0.82, P<0.001, I^2=43%; GRADE = low), but greater tolerability did not affect withdrawal rate.

Oral corticosteroids were compared to 5-aminosalicylic acid (5-ASA) in eight studies of which 3 were rated as of low risk of bias (n = 1114). There was no difference in clinical remission, clinical response or endoscopic remission rates between the two agents. Patients treated with oral corticosteroids, however, were less likely to achieve histological remission compared to patients treated with 5-ASA (3 studies, n = 888 patients RR 0.67 95% CI 0.54-0.83; P<0.001; I^2=77%; GRADE = very low).

Five trials (3 with a low risk of bias) compared a higher to a lower dosed regimen of oral corticosteroids (N=801) and demonstrated higher dosing to be more effective for achieving clinical remission and response (respectively, 5 trials, 800 patients, RR 1.56, 95% CI 1.17 to 2.08, P=0.002, I^2=84%; GRADE = very low and 4 trials, 680 patients, RR 1.44, 95% CI 1.02-2.02, P=0.04, I^2= 0%, GRADE = low). High dose corticosteroids also induced endoscopic remission more often than lower dosed regimens (2 trials, 576 patients, RR 1.27 95% CI 1.01-1.60, P=0.04, I^2=0%; GRADE = low), however no differences in histological response were detected. Higher dose strategies were not associated with more frequent adverse events (5 trials, 801 patients, RR 0.96 95% CI 0.86-1.08, P=0.50, I^2=0%; GRADE = moderate).

Two other, open label studies with a high risk of bias compared oral corticosteroids to another active comparator (tumour necrosis factor (TNF) antagonists or antibiotics) and found no differences in clinical remission. One study (open label, high risk of bias, N= 120) compared oral administration of corticosteroids to topical corticosteroid enemas and found the topical treatment to be more effective in inducing clinical remission with clinical remission achieved in 14/40 (35%) of patients treated with oral corticosteroids vs 29/40 (73%) treated with corticosteroid enemas (1 trial, 80 patients, RR 0.48 95% CI 0.30-0.70, P=0.002).

Authors' conclusions. In this systematic review and meta-analysis oral corticosteroids were found to lead to significantly greater clinical, endoscopic and histological remission as compared to placebo, without significantly more adverse events or withdrawals due to adverse events. Additionally, locally active corticosteroids were also able to induce clinical, but appeared less effective in inducing endoscopic remission compared to systemic corticosteroids, although they were associated with less frequent adverse events. In conclusion, this meta-analysis provides evidence to support the use of oral corticosteroids (both systemic and locally active) for the induction of remission in active UC.

BACKGROUND

Inflammatory bowel diseases (IBD) are a group of immune-related disorders primarily targeting the gastro-intestinal system, com- prised of two main forms: Crohn's disease (CD) and ulcerative colitis (UC). These diseases share similarities in the underlying pathophysiology, yet have important differences in both presentation and approach to treatment. Although the exact cause of IBD remains unclear, genetic factors, microbial disturbances and ab- normal host immune reactions play important roles (Ugaro 2017).

Description of the condition

Ulcerative colitis mainly affects the mucosal layers of the colon and is characterized clinically by bloody diarrhoea, abdominal pain or cramping, and tenesmus. Many patients experience a clinical course marked by periods of exacerbation and remission. Inflammation usually starts at the rectum and extends continuously to the proximal colon. UC is subcategorised based on disease extent with most of adult patients presenting with a left-sided colitis. Anatomic location has important consequences for the management of the disease (Ugaro 2017), given that disease distal to the splenic flexure may be managed with topical therapy in the form of enemas, foams or suppositories. At present, there is no definite cure available for UC. Treatment is aimed at inducing and maintaining remission and preventing disease complications such as hospitalisation and surgery.

Therapeutic options in UC management depend on severity and extent of the disease. First line therapy is primarily based on 5-aminosalicylates (5-ASA) and corticosteroids (Magro 2017). Re- fractory disease is managed by addition of immunosuppressants (azathioprine) or Tumor necrosis factor-alpha (TNFα) antagonists. Alternatively, vedolizumab, an α4β7 Integrin antagonist which halts leukocyte trafficking to the gut, is effective in both inducing and maintaining remission. Several other agents are currently entering phase III trials (Neurath 2017). For patients with severe ongoing inflammation, refractory to conventional medical therapy colectomy is an effective alternative solution, especially when rescue therapy with infliximab or cyclosporine fails. (Magro 2017)

Description of the intervention

Corticosteroids have been used in the management of UC for decades. The efficacy of corticosteroids was first documented by Dearing 1950, and subsequently observed in several other observational studies (Kirsner 1950, Machella 1951; Milanes Alvarez 1951; Whiteside 1951). A highly influential randomised con- trolled trial (RCT) conducted by Truelove 1955 demonstrated the short term benefit of corticosteroids over placebo and they have been used in the management of UC patients ever since.

Current guidelines recommend corticosteroid use when treatment with 5- ASA has been unsuccessful (Magro 2017). Corticosteroids are available in oral and topical formulations. Several oral products are available ranging from agents with predominantly systemic actions (systemic corticosteroids) to second generation corticosteroids with a more localized pharmacodynamic effect (locally active corticosteroids).

Oral corticosteroids with systemic actions such as prednisolone, methylprednisolone or dexamethasone are superior to placebo in inducing remission in UC patients (Ford 2011), however they are associated with important adverse events. Over 90% of patients will experience adverse effects ranging from mild skin alterations to life-threatening infections (Lichtenstein 2006). The most frequently reported side-effects which occur with even low- to-medium dose corticosteroids are mood alterations, other psychological disturbances and peptic ulcers (Curkovic 2013; Curtis 2006). Other adverse events associated with prolonged systemic corticosteroid use are glaucoma, cataract formation, development of a cushingoid appearance, metabolic complications (e.g. hyper- glycaemia, diabetes and weight gain) and alterations in bone formation leading to osteoporosis and an increased hip fracture risk (Card 2004; Lennard-Jones 1960; Lennard-Jones 1965; Patten 2000; Vakil 1989).

Newer locally active corticosteroids such as budesonide, beclomethasone dipropionate, fluticasone propionate and prednisolone metasulfobenzoate were developed to target intestinal inflammation locally with only limited systemic exposure to the drug in the hope of reducing the risk of side-effects. All of these locally active oral corticosteroids undergo an extensive hepatic first- pass metabolism which results in a low systemic bio-availability (D'Haens 2016). To specifically target delivery to the ileum and colon, most of the products make use of dedicated drug distribution technologies with the exception of fluticasone propionate which reaches the colon in sufficient concentrations exclusively due to poor proximal absorption (Hawthorne 1993). Prednisolone metasulfobenzoate (Predocol®) is delivered specifically to the dis- tal colon by use of the pH-sensitive Eudragit capsule coating tech- nology (Evonik Industries, Essen, Germany) (Rhodes 2008), while beclomethasone dipropionate (Clipper®, Chiesi, Italy) is released at a pH ≥ 6.4 due to a methacrylic polymer coating securing its distribution throughout the colon. (Rizzello 2002). Several enteric release formulations are available for budesonide (Entocort® (AstraZeneca, UK) and Budenofalk® (Dr. Falk Pharma, Germany). Entocort® uses a methylcellulose matrix which releases the drug in environments with a pH above 5.5 (e.g. small intestine), while budesonide resides in microgranules releasing active product at a pH ≥ 6.4 (Prantera 2013). More recently, a multi matrix system (MMX) technology was developed (MMX, Cosmo Technologies, Dublin, Ireland) that allows for controlled release throughout the entire colon (Budesonide MMX, Urceris®, Salix Pharmaceuticals, USA) (Danese 2014).

How the intervention might work

Corticosteroids are known to suppress lymphocyte activation and proliferation through inhibition of several inflammatory pathways including NFkB activation and subsequent transcription of interleukin-1, interleukin-6 and TNFα. Additionally they interfere with both granulocyte and macrophage function and arachidonic acid metabolism (Franchimont 2003).

Corticosteroid-induced adverse events make the locally active second-generation corticosteroids such as budesonide attractive alternatives to traditional systemic corticosteroids. The latter agents result in less exposure of the hypothalamic-pituitary axis to drug and potentially less systemic side effects.

Why it is important to do this review

Considerable heterogeneity exists among clinicians in their use of corticosteroid regimens for treating active UC due to a lack of clarity and over-perceived differences between efficacy and safety of systemic and locally active corticosteroids. Further uncertainty exists regarding optimal initial dosing, tapering and duration of treatment. This review aims to systematically evaluate the efficacy and safety of corticosteroids used for induction of remission in UC in the interest of facilitating evidence-based decision making.

OBJECTIVES

The primary objective was to evaluate the efficacy and safety of oral corticosteroids used for induction of remission in active UC.

Secondary objectives were evaluating efficacy and safety for induc-ing remission in UC between

1. Systemic corticosteroids and locally active agents
2. High dose and lower dosed corticosteroid regiments
3. Oral corticosteroids and other active treatments
4. Oral corticosteroids and topical formulations

METHODS

Criteria for considering studies for this review Types of studies

RCTs comparing oral corticosteroids versus placebo or an active comparator for the treatment of active UC (whether first attack or relapse) were considered for inclusion.

Types of participants

Patients of any age with active UC, as defined by a combination of clinical, radiographic, endoscopic and histologic criteria were considered for inclusion.

Types of interventions

RCTs in which patients were treated with oral corticosteroids, placebo or an active comparator (e.g. 5-ASA) were considered for inclusion.

Types of outcome measures

The primary outcome measure was the number of patients achieving clinical remission as defined by the individual studies, and ex- pressed as a percentage of the patients randomised (intention-to- treat analysis).

Secondary outcomes were:(1) endoscopic remission;(2) clinical, endoscopic and histological improvement;(3) change in Ulcerative Colitis Disease Activity Index (UCDAI) (clinical symptoms and endoscopic appearance of mucosa);(4) treatment failure requiring additional treatment;(5) surgery (colectomy);(6) adverse events; and(7) withdrawal due to adverse events.

Search methods for identification of studies

The following databases were searched:1. PubMed (August, 9, 2016)2. EMBASE (1947 to August 9, 2016; Elsevier Science, New York, USA);3. MEDLINE (1946 to August 9, 2016; National Library of Medicine, Bethesda, USA);4. The Cochrane Central Register of Controlled Trials (CEN- TRAL, August 2016); and5. The Cochrane Inflammatory Bowel Disease and Functional Bowel Disorders Group Specialized Trials Register (SR-IBD). The search was not limited by language. The search strategies are reported in Appendix 1. Reference lists from relevant papers will be scanned to identify additional

citations.

Data collection and analysis Selection of studies

Two authors (TH and WV) independently assessed the eligibility of potentially relevant studies based on the aforementioned inclusion criteria. Disagreements among authors were resolved by consensus.

Data extraction and management

Two authors (TH and WV) independently abstracted the results of eligible studies using a data extraction form. The following information was recorded:

a) Number of patients enrolled, number of patients per treatment arm (to allow for an intention-to-treat analysis);
b) Type of intervention: dose, frequency, duration, form, number of patients on oral corticosteroid or placebo or other active agents;
c) Patients characteristics: age, sex distribution, disease extent and duration;
d) Outcomes: number of patients completing treatment, number of dropouts due to adverse effects events. Number of patients improved or entered into remission by symptomatic, histologic and endoscopic criteria. Where possible, the median number of days to remission and the mean change in disease activity index
e) Definitions: clinical improvement, endoscopic improvement, histologic improvement, clinical remission, endoscopic remission and histologic remission. Definitions of 'remission', 'improvement', and 'relapse' were expected to vary among studies. The original authors' definitions of these outcomes were used.

Assessment of risk of bias in included studies

Two authors (TH and WV) independently evaluated the methodological quality of each study using the Cochrane risk of bias tool (Higgins 2011). Factors assessed included:
1. sequence generation (i.e. was the allocation sequence adequately generated?);
2. allocation sequence concealment (i.e. was allocation adequately concealed?);
3. blinding (i.e. was knowledge of the allocated intervention adequately prevented during the study?);
4. incomplete outcome data (i.e. were incomplete outcome data adequately addressed?);
5. selective outcome reporting (i.e. are reports of the study free of suggestion of selective outcome reporting?); and
6. other potential sources of bias (i.e. was the study apparently free of other problems that could put it at a high risk of bias?). Based on these characteristics, publications studies were judged to have a

low, high or unclear risk of bias. Disagreements among review authors were resolved by consensus. Study authors were contacted if there was inadequate information to determine the risk of bias.

The GRADE criteria were used to assess the overall quality of evi- dence for the primary outcomes of interest. RCTs were considered high quality evidence, however, they may have been downgraded due to: (1) limitations in design and implementation (risk of bias), (2) indirectness of evidence, (3) inconsistency (unexplained het- erogeneity), (4) imprecision (sparse data), and (5) reporting bias (publication bias). After considering each of these elements, the overall quality of evidence for each outcome was rated as high quality (i.e. further research is very unlikely to change our confidence in the estimate of effect); moderate quality (i.e. further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate); low quality (i.e. further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate); or very low quality (i.e. we are very uncertain about the estimate) (Guyatt 2008; Schünemann 2011).

Measures of treatment effect

The Cochrane Collaboration Review Manager software (RevMan 5.2) was used for data analysis. All data were analysed on an intention-to-treat basis. For dichotomous outcomes, the relative risk (RR) and corresponding 95% confidence intervals (95% CI) were calculated for dichotomous outcomes. The mean difference (MD) and corresponding 95% CI were calculated for continuous out- comes.

Dealing with missing data

Since the primary analysis was carried out on an intention-to-treat basis, missing data were assumed to be treatment failures.

Assessment of heterogeneity

Heterogeneity among studies was assessed using the Chi^2 and I^2 statistics (Higgins 2003). A P value of 0.10 was considered to be statistically significant.

Assessment of reporting biases

An insufficient number of relevant studies prevented the investigation of potential publication bias using funnel plots (Egger 1997).

Data synthesis

Data were pooled for analysis if patients, interventions and out- comes across studies were

determined to be sufficiently similar. Studies were not pooled for meta-analysis if a high degree of het- erogeneity (e.g. I^2 > 75%) was found. The pooled RR and corresponding 95% CI were calculated for dichotomous outcomes. A fixed-effect model was used to pool data when statistical hetero- geneity was not present. When statistical heterogeneity was present (i.e. statistically significant Chi^2 test and I^2 is > 50%), a random- effects model was used. The pooled MD and corresponding 95% CI were calculated for continuous outcomes.

Subgroup analysis and investigation of heterogeneity

When possible, subgroup analyses were performed based on the following:

a. disease distribution (distal, left-sided or total colitis);

b. type of oral corticosteroid;

c. dose of oral corticosteroid;

d. duration of treatment;

e. concurrent immunosuppressants (azathioprine, 6-mercaptopurine, methotrexate, cyclosporine) and TNF antagonists

f. length of follow up.

RESULTS

Description of studies

Results of the search

A literature search conducted on August 9 2016 identified 2,885 studies. After duplicates were removed a total of 2,010 studies remained for review of titles and abstracts. Two authors (TH and WV) independently reviewed these publications and 90 studies were selected for full text review (See Figure 1). Two authors (TH and WV) then reviewed the full-text articles. Fifty-one of these studies were excluded (See Characteristics of excluded studies). Twenty-three studies were selected for inclusion in this review.

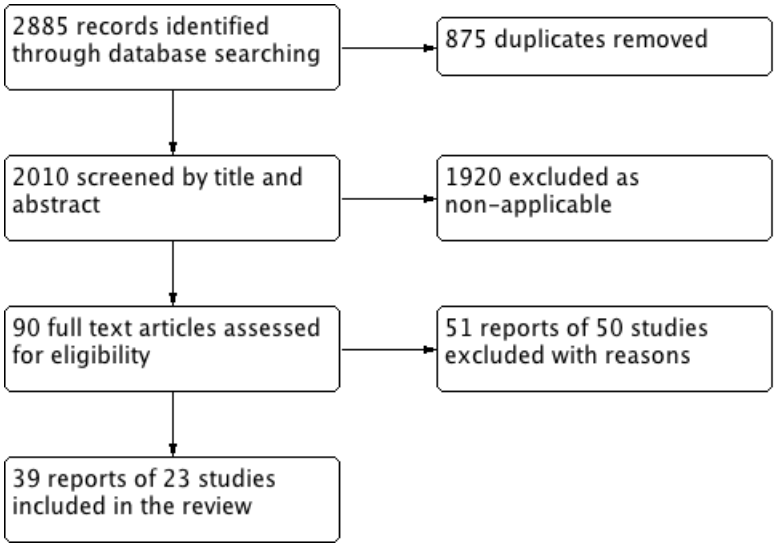

Figure 1 – Flow diagram

The reference lists of main review articles were searched to identify any additional studies not identified by the primary search strategy (Baumgart 2007; D'Haens 2016; Danese 2014; De Cassan 2012; Ford 2011; Gionchetti 2014; Hoy 2015, Kozuch 2008; Magro 2017; Silverman 2011) however no additional studies were identified for inclusion.

Included studies

Twenty-three trials including a total of 3338 patients were identified for inclusion in this review. Seven studies compared oral cor- ticosteroids with placebo (Angus 1992; D'Haens 2010; Rizzello 2002; Rubin 2015; Sandborn 2012; Travis 2014; Truelove 1955), four systemic corticosteroids to locally active products (Löfberg 1996; Hawthorne 1993, Rhodes 2008, Van Assche 2015), ten studies compared oral corticosteroids to an active comparator (TNF antagonists: Armuzzi 2004; antibiotics: Bataga 2015; 5-ASA: Campieri 2003; Gross 2011; Lechin 1985; Lennard-Jones 1960; Pica 2013; Raj 2014; Romano

2010; Sandborn 2012), four studies compared high dose oral corticosteroids and low dose (Baron 1962; Powell-Tuck 1978; Sandborn 2012; Travis 2014) while only one study directly compared oral and topical corticosteroids (Truelove 1960a).

Different formulations of oral corticosteroids were used in the included studies: in 8 studies a systemic corticosteroid was used (methylprednisolone: Armuzzi 2004; Prednisolone: Baron 1962; Lechin 1985; Löfberg 1996; Powell-Tuck 1978; Raj 2014; Truelove 1960a; cortisone: Truelove 1955), while the others used a locally active corticosteroid preparation (6 studies used budesonide (Bataga 2015; Gross 2011; budesonide MMX formulation D'Haens 2010; Rubin 2015; Sandborn 2012; Travis 2014); 2 fluticasone dipropionate (Angus 1992; Hawthorne 1993), in 5 stud- ies beclomethasone dipropionate was used (Campieri 2003; Pica 2013; Rizzello 2002; Romano 2010; Van Assche 2015) and 1 study examined prednisolone metasulfobenzoate (Rhodes 2008)).

STUDIES COMPARING ORAL CORTICOSTEROIDS TO PLACEBO

Angus 1992 conducted a randomised, single-centre, placebo-con- trolled trial allocating patients with mild or moderately active UC that did not extend beyond the splenic flexure to fluticasone propionate 20 mg given orally in 5 mg doses four times daily (n = 30) or matched placebo (n = 29) for four weeks. Entry was restricted to patients (aged 18-65 years) who were systemically well and passing more than three but less than seven motions daily. Patients receiving sulphasalazine, Mesalazine or olsalazine prior to relapse were permitted to continue treatment. Patients receiving active corticosteroids within two weeks of study initiation were excluded. Outcomes were clinical, endoscopic and histological response after four weeks of treatment. At the end of this period, there was no significance difference in response between the fluticasone propionate and placebo groups. While the authors conclude that fluticasone propionate (5 mg four times daily) is an ineffective treatment for the induction of remission in active distal UC, there were an insufficient number of patients enrolled in the study to make this claim.

D'Haens 2010 conducted a randomised, multi-center, double- blind, placebo-controlled trial allocating patients with mild to moderately active left-sided UC that did not extend beyond the splenic flexure to Budesonide-MMX 9 mg once daily (n = 18) or placebo (n = 18) for 4 weeks. Patients with worsening disease after two weeks or failure to enter remission after four weeks received open-label budesonide for an additional 4 to 6 weeks. Entry was restricted to patients receiving oral 5-ASA (between 0 to 3g/day) for at least two months prior to enrolment with a colitis activity index (CAI) score < 14. Patients receiving immunosuppressive drugs (with the exception of 6-mercaptopurine and azathioprine), or

corticosteroids within one month prior to study initiation were excluded. The primary outcome was the number of patients achieving clinical improvement (meaning remission as de- fined as CAI ≤4; or improvement as defined as a CAI reduction by at least 50% of the baseline value). Secondary outcomes included a reduced CAI by at least 70%, clinical remission after 8 weeks (6 weeks in the case of patients switched to open-label budesonide due to worsening disease), and endoscopic and histological assessment. After 4 weeks clinical improvement was achieved in 47.1% and 33.3% of the Budesonide-MMX and placebo groups respectively. The same proportion of each group experienced improvement without remission. CAI reduction was significant with Budesonide-MMX (P = 0.0001) and not with placebo (P = 0.1). Measures of hypothalamic - pituitary - adrenal axis function were not appreciably different in the two groups. The authors conclude that Budesonide-MMX® 9 mg tablets can effectively and safely induce significant clinical improvement and remission in patients with active left-sided UC over a 4 week treatment period.

Rizzello 2002 conducted a randomised, multi-center, double- blind, placebo-controlled trial allocating patients with mild to moderately active UC to oral beclomethasone dipropionate (BDP) 5 mg/day plus oral 5-ASA 3.2 g/day (n = 58) or placebo (n = 61) for 4 weeks. Entry was restricted to patients (aged 18 years and older) with extensive or left-sided UC and a disease activity index (DAI) score of 3 to 10 points. Patients with severe UC (DAI >10), a new diagnosis or those in clinical remission were excluded from the study. In addition, patients receiving corticosteroids within 1 month prior to study initiation, or 5-ASA (> 3.2 g/day) or sulphasalazine (> 2 g/day) for 2 weeks prior to study initiation were also ineligible. The primary outcomes were changes in daily stool frequency, blood in stools, subjective sense of well-being and mucosal appearance at colonoscopy. Secondary outcomes included histology, erythrocyte sedimentation rate (ESR), clinical remission, morning serum cortisol levels and pituitary-adrenal function and adverse events. At the end of week 4, there was a significant decrease of disease activity index (DAI) and histology score and low incidence of adverse events in both the BDP plus 5-ASA (P = 0.001) and placebo groups (P = 0.001). However, the DAI score was lower (P = 0.014) and more patients achieved clinical remission in the BDP group compared to placebo (58.6% vs. 34.4%, P = 0.008). Although serum cortisol concentrations significantly decreased from baseline in the BDP group versus baseline (P = 0.002), this was not associated with clinical manifestations signs of pituitary-adrenal function depletion. The authors concluded that oral BDP in combination with oral 5-ASA is significantly more effective than 5-ASA alone in the treatment of patients with extensive or left-sided active UC.

Rubin 2015 performed a single centre, randomised, double blind, placebo-controlled trial including

UC patients with mild to moderately active disease (UCDAI >4 and < 10) inadequately controlled with 5-ASA. Patients with normal baseline histology or infectious colitis were excluded from the trial. Included patients were randomised to treatment with budesonide MMX 9 mg (n= 230) or placebo (n=228) for a duration of 8 weeks. Patients were required to be receiving a stable, therapeutic dose of an oral 5-ASA (e.g., Mesalazine >2.4 g/day or equivalent dose of another 5-ASA) throughout the study. Primary outcomes were combined clinical and endoscopic remission at week 8, as defined by a UCDAI score of <1, with subscores of 0 for rectal bleeding, stool frequency and mucosal appearance. Secondary and exploratory endpoints assessed clinical remission (rectal bleeding and stool frequency sub- scores = 0), endoscopic remission (mucosal appearance subscore = 0) and histological healing (histological activity grade = 0, as assessed via central reading). Combined clinical and endoscopic remission was achieved in 13% of the budesonide treated patients vs. 7.5% in the placebo group (P=0.0488). Endoscopic remission was achieved by 20% in the budesonide group vs. 12.3% in the placebo group (P = 0.0248), budesonide also induced histological healing in a greater percentage of patients than placebo (27% vs. 17.5%, P = 0.0155). Adverse event rates were similar between groups (31.8% in the budesonide group vs 27.8% placebo, discontinuation due to adverse events in 4.7% and 3.5%, respectively. The authors concluded that addition of budesonide MMX 9 mg in patients experiencing an active flare of UC despite baseline oral 5-ASA therapy was significantly more effective than placebo at inducing combined clinical and endoscopic remission as well as histological healing.

Sandborn 2012 conducted a randomised, multi-center, double- blind, double-dummy, placebo-controlled trial that allocated pa- tients with active UC to one of 4 treatments: oral budesonide MMX (B-MMX) 9 mg once daily (n = 127), oral budesonide MMX 6 mg once daily (n = 128), oral Mesalazine 2.4 g/day (n = 127; administered as two 400-mg tablets 3 times daily) or matched placebo for 8 weeks. Entry was restricted to patients (aged 18- 75 years) diagnosed with mild to moderately active UC within 6 months and an Ulcerative Colitis Disease Activity Index (UC- DAI) score of 4 to 10 points. Patients were excluded if they had received oral or rectal corticosteroids within 4 weeks of screening, TNF antagonists within 3 months of screening, participated in another therapeutic study within 3 months of screening or were diagnosed with severe UC (UCDAI > 10). The primary outcome was combined clinical and endoscopic remission at week 8. Secondary outcomes included clinical improvement (defined as a > 3 point reduction in UCDAI), endoscopic improvement (defined as a > 1 point reduction in the mucosal appearance score of the UC- DAI), histologic healing (defined as a score of < 1 on the Saverymuttu scale), symptom resolution (defined as a score of 0 for both rectal bleeding and stool frequency sub-scores from the UCDAI) and adverse events. At the end of week 8 a significantly greater number of patients in the B-MMX 9 mg group achieved remission compared to

placebo in both the modified intent-to-treat (ITT) population (17.9% vs. 7.4%; P = 0.0143) and in analysis of all randomised patients (17.3% vs. 7.0%; P = 0.0119). Statistical significance was not observed for the B-MMX 6 mg group to placebo comparison. No significant differences were observed between the study groups in the frequency of treatment-related adverse events and serious adverse events. The authors concluded that B-MMX 9 mg administered once daily was safe and effective at inducing remission in patients with mild to moderate UC.

Travis 2014 conducted a randomised, double-blind, double- dummy, placebo-controlled, parallel-group trial allocating patients with active UC (up to the splenic flexure) to B-MMX 9 mg(n=126),B-MMX6mg(n=128),EntocortEC9mg(n= 126; given in 3 mg doses three times daily) or placebo (n = 129) for 8 weeks. Entry was restricted to patients (aged 18-75 years) diagnosed with mild to moderately severe UC within 6 months and a UCDAI score of 4 to 10 points. Concomitant therapy for UC was not permitted; patients receiving oral or rectal 5-ASAs at the screening visit required a 2 or 4-week washout prior to randomisation, respectively. Patients were excluded from the study if they had received oral or rectal corticosteroids within 4 weeks of screening, immunosuppressive agents within 8 weeks of screening, anti-tumor necrosis factor agents within 3 months of screening or participated in another experimental therapeutic study within 3 months of screening. Patients with severe UC (UCDAI > 10) were also excluded. The primary outcome was combined clinical and endoscopic remission at week 8 (defined as a total UCDAI score ≤1, with a rectal bleeding score of 0, stool frequency score of 0, mucosal appearance score of 0 and a ≥1-point reduction in baseline endoscopic index score) at week 8. Secondary outcomes included clinical improvement (defined as a ≥3 point reduction in UCDAI score), endoscopic improvement (defined as a ≥1-point reduction in the endoscopy sub-score of the UCDAI), symptom resolution (defined as a score of 0 for both rectal bleeding and stool frequency sub-scores from the UCDAI) and histologic healing (defined as a score of < 1 on the Saverymuttu scale). At the end of the treatment period B-MMX 9 mg was associated with numerically higher rates of clinical (42.2% vs 33.7%) and endoscopic (42.2% vs 31.5%) improvement versus placebo. A significant number of patients in the B-MMX 9 mg group achieved combined clinical and endoscopic remission (17.4% vs 4.5%; P = 0.0047), improved histological healing (16.5% vs 6.7%; P = 0.0361) and symptom resolution (23.9% vs 11.2%; P = 0.0220) compared to placebo. Adverse events occurred at similar frequencies among groups. The authors concluded that B-MMX 9 mg was safe and more effective than placebo at inducing remission in patients with mild to moderate UC.

Truelove 1955 conducted a randomised, double-blind, multi-cen- ter, placebo-controlled trial allocating patients with UC to cortisone (n = 109) or placebo (n = 101) for 6 weeks. In the cortisone

group, 38 patients received 100 mg/day for 6 weeks, 38 patients received 100 mg/day for 2 to 3 weeks followed by smaller doses of 50-75 mg/day, 17 patients received > 100 mg/day and 16 patients received therapy for less than the treatment period. Entry was restricted to patients with first attack or relapse of mild to severe UC. Diagnosis was based on history, character of stools, sigmoidoscopy (proctoscopy was regarded as sufficient in patients with severe disease), barium enema (except in patients with severe dis- ease), and absence of known pathogens in the stools. Patients diagnosed with regional colitis, ileitis or proctitis were excluded. Out- comes included clinical remission (defined as one or two stools/ day without blood, no fever, no tachycardia, normal haemoglobin and normal erythrocyte sedimentation rate), clinical improvement, sigmoidoscopic appearance (defined as normal, near nor- mal, improved, no change or worse), barium enema findings and complications. At the end of the treatment period a significantly greater number of patients in the cortisone group achieved clinical remission, clinical improvement, and improved sigmoidoscopic appearance compared to placebo. Complications occurred at similar frequencies among groups. The authors concluded that cortisone therapy safely enhanced the chance of achieving remission or improvement in patients with mild to severely active UC.

STUDIES COMPARING SYSTEMIC TO LOCALLY ACTIVE CORTICOSTEROIDS

Hawthorne 1993 conducted a multi-center, randomised, double-blind placebo-controlled trial including UC patients with active disease with left-sided or pancolitis, were outpatients and had at least three bowel actions a day passing liquid or semi formed stools. Patients with Crohn's disease, pregnancy or concomitant serious medical conditions were not considered for this trial. Included patients were treated with oral prednisolone 40 mg (n=105) or fluticasone dipropionate 5 mg 4 times daily (n = 101). Primary outcomes were clinical remission and response as assessed by the treating physician as well as changes in the sigmoidoscopy score after 28 days of treatment. Remission rates at 4 weeks did not differ significantly between the prednisolone group (29%) and fluti- casone group (26%), although patients in the prednisolone group obtained remission faster and had better specific symptoms scores (less rectal blood loss, less frequent stools). Corticosteroid-related adverse events were more common in the prednisolone group (7% vs 0% in the fluticasone group). Based on these results the authors concluded that prednisolone is superior for inducing remission in active UC as compared to fluticasone, although the latter resulted in less corticosteroid-related adverse events and suppression of adrenal function. Usage of a higher dosage of fluticasone was proposed for further studies.

Löfberg 1996 performed a multicenter, randomised, double- dummy, double-blinded controlled trial

in UC patients with mild to moderately active disease extending beyond the sigmoid colon who were allocated to either treatment with prednisolone (n = 38) or budesonide (n = 34) for a period of 9 weeks. Primary outcomes were endoscopic and/or histological remission at week 4, secondary outcomes were endoscopic and/or histological response at week 9. At week 4, 13% of patients treated with budesonide reached endoscopic remission compared with 17% in the prednisolone group (non-significant (NS)). Histological remission rates were similar in both treatment groups (budesonide group 10% vs 17% in the prednisolone group). There were no differences in response rates at 9 weeks. Adverse events were similar between both groups, however patients in the budesonide group had less suppression of the morning cortisol levels than those who received prednisolone. The authors concluded that budesonide was equally effective as prednisolone without causing hypothalamic-pituitary-adrenal axis (HPA) suppression as defined by morning cortisol concentrations.

Rhodes 2008 performed a randomised, multi-center, double- blind, placebo controlled trial in UC patients with active dis- ease extending to at least the descending colon who did not meet the criteria for severe colitis (Truelove & Witts < 10), were not treated with corticosteroids in the last month, were not receiving azathioprine or 5-ASA maintenance therapy for at least three months and who were not corticosteroid refractory. Included patients were randomised to treatment with the locally active corticosteroid Predocol 40 mg (n=59) or 60 mg (n=61) or otherwise conventional prednisolone 40 mg in tapering dose (n=61) for a period of 6 months. Primary outcomes were global visual analogue scale (VAS) assessment of symptoms and side-effects, secondary outcomes were clinical remission (Powel-Tuck ≤ 2), endoscopic remission (Barron score ≤ 1). VAS assessed corticosteroid-related adverse events were fewer at 2 months with Predocol 40 mg [VAS 8.1 cm (2.6), mean (S.D.)], or 60 mg [8.1 (2.1)] compared with prednisolone [6.7 (2.7); P = 0.01]. Mood changes affected 43% receiving prednisolone at 4 weeks vs. 8% for Predocol 40 mg (P = 0.001). Remission rates at 2 months were Predocol 40 mg 46%, Predocol 60 mg 28% and tapering prednisolone 41% (P = 0.13). Visual analogue scale for efficacy also showed similar results be- tween groups. Remission rates at 6 months were Predocol 40 mg 51%, Predocol 60 mg 38% and tapering prednisolone 32% (P = 0.08). The authors concluded that Predocol 40 mg was equally effective as tapering prednisolone with fewer side-effects.

Van Assche 2015 conducted a multi-center, double blind, randomised controlled trial in patients with UC and a DAI score of ≥1 who were on a stable dose of 5-ASA (if any) for the last two weeks. Exclusion criteria were use of corticosteroids in the last 30 days, treatment with immunosuppressants in the last 3 months and TNF antagonists in the last 6 months. These medications were also not permitted during the study. Patients with severe colitis (DAI > 9) or those with important co-morbidities were also

excluded. Patients were allocated to treatment with either prednisone in tapering dose (n = 145) or beclomethasone dipropionate (BDP) (n=137) for a total period of 4 weeks. Primary outcomes were clinical response (defined as a drop in DAI of ≥ 3) and ad- verse events at 4 weeks of treatment. Secondary endpoints were clinical remission (DAI ≤ 1) and endoscopic remission (score 0- 1). At the end of the trial, 22.8% of patients were in clinical remission in the corticosteroid group as opposed to 19.2% in the BDP treated patients, while clinical response was achieved in respectively 66.2% and 64.6% of patients respectively (P=0.78). Endoscopic remission rates were similar between the two groups (21.5% in the corticosteroid group vs 23.3% in the BDP treated patients). Patients with corticosteroid-related AEs and plasma cortisol concentrations <150 nmol/l at week 4 were 38.7% in the BDP group and 46.9% in the PD group (P=0.17 between groups). The authors concluded that BDP was non-inferior to prednisone for the treatment of active UC.

STUDIES COMPARING ORAL CORTICOSTEROIDS TO ACTIVE COMPARATORS

Armuzzi 2004 conducted a randomised, single-center, open label trial allocating patients with moderate to severe corticosteroid- dependent UC to oral methylprednisolone 0.7-1 mg/kg (n=10) or intravenous infliximab 5 mg/kg (n=10) for 54 weeks. Entry was restricted to patients with a disease activity index (DAI) > 6 and who were continuously treated with corticosteroids for at least one year. Outcomes were clinical remission and changes in DAI and HRQL after 54 weeks of treatment. At the end of the trial there were no significant differences between treatment groups leading to the author's conclusion that infliximab and corticosteroids were equally effective for the management of corticosteroid-refractory moderate to severe UC.

Bataga 2015 conducted a randomised, single-center, open label trial treating patients with a mild relapse of left-sided UC with either budesonide 9 mg daily (n =24) administered for 3 months in a tapering dose or rifaximin 800 mg once daily for 1 month followed by a probiotic (Lactobacillus acidophilus, Bifidobacterium infantis and Enterococcus faecium) for 10 days (n=24). Treatment with a stable dose of 5-ASA was required. The primary outcomes were clinical, endoscopic and histological remission at 6 months. No significant differences were observed in remission rates between the budesonide and rifaximin group. Based on these results the authors concluded that rifaximin 800 mg a day for 1 month followed by a probiotic is an efficient strategy for mild UC flares. This study was only published as an abstract.

Campieri 2003 performed a multi-center, single blinded randomised clinical trial in which patients with mild to moderate UC were assigned to either beclomethasone dipropionate 5 mg/day (n = 90) or 5-

ASA (Asacol®) 0.8 g 3 times a day (n = 87). Only patients with extensive disease or left-sided colitis with a DAI be- tween 3 and 10 were included. Those in remission or with a severe colitis (DAI > 10), patients with important co-morbidities or treatment with corticosteroids or 5-ASA within one month of inclusion were not eligible. Primary outcomes were clinical remission, clinical response, changes in DAI and changes in morning cortisol levels, secondary outcomes included changes in clinical symptoms and inflammatory parameters (WBC, CRP, ESR). After 4 weeks of treatment there was no statistically significant difference in clinical remission (63% in the budesonide group vs 62.5% in the 5-ASA group) or clinical response rates, although a significantly greater drop in DAI in the budesonide group was observed in comparison to the placebo group. Morning cortisol levels were significantly lower in the budesonide group however no clinical manifestations of HPA axis insufficiency were observed. The authors concluded that budesonide was not inferior to 5-ASA in active UC and was
not associated with systemic corticosteroid-related adverse events.

Gross 2011 conducted a multi-center, randomised, double-blind controlled trial in which patients with mild to moderately active UC were allocated to treatment with budesonide 9 mg once daily (OD) (n= 177) or Mesalazine 3000 mg OD (n = 166). Active disease was defined by a CAI ≥ 6 and endoscopic index (EI) ≥ 4. Patients with proctitis, other causes of colitis or previously treated with immunosuppressants (< 3 months prior to enrolment), corticosteroids (< 4 weeks prior to enrolment) or who had a relapse under Mesalazine maintenance therapy were excluded from the trial. The primary outcome was clinical remission after 8 weeks of treatment (defined as CAI ≤ 4 with stool frequency and rectal bleeding sub scores of 0), secondary outcomes were endoscopic remission (defined as EI ≤ 3 with mucosal healing ≤1), histological remission, endoscopic or histological response (more than 1 point drop in respective score). At the end of the trial, fewer patients achieved clinical remission in the budesonide group (39.5%) than those assigned to Mesalazine (54.8%). Mucosal healing was observed in similar proportions in the budesonide and Mesalazine group (30.5% vs 39.2%, respectively). The incidence of adverse events and serious adverse events was similar between both groups. The authors concluded that Mesalazine was superior to budesonide in the treatment of acute mild to moderately active UC.

Lechin 1985 performed a single centre, randomised, double blind controlled trial in UC patients with a severe flare of the disease (10 or more bloody stools a day) who had not been treated pre- viously with corticosteroids or sulphasalazine in the prior three months. Patients were allocated to treatment with prednisolone 20 mg (n = 15), clonidine 0.3 mg (n = 15) or sulphasalazine 1.5 mg three times daily (n=15). Patients were treated for 3 six-week periods separated by 2 six-week placebo periods for a

total duration of 30 weeks. Primary outcomes were clinical, endoscopic and histological response measured by 5-point rating scales after every treatment period. Secondary outcome was response on radi- ological imaging. After the first induction period 67% of patients treated with prednisolone achieved clinical response as opposed to 47% with sulphasalazine.

In a single centre randomised, open label, placebo-controlled study by Lennard-Jones 1960 UC patients with mild active disease with extent to the splenic flexure were included. In a first part of the study patients were allocated to prednisone in tapering dose (n= 19) or placebo (n=18), in the second part patients were treated with prednisone in tapering dose (n = 20), sulphasalazine (n = 20) or hydrocortisone hemisuccinate enemas (n=20) for a period of 3 weeks. The primary outcome was clinical and endoscopic re- mission at week 3. Prednisolone was more effective in inducing remission than placebo (68% vs 13%). In the second part of the study, remission was obtained by 50% of patients in the pred- nisone group, 40% in the sulphasalazine treated patients and 15% in the patients treated with hydrocortisone enemas. The authors concluded that prednisone performed better in inducing remis- sion in patients with mild, active UC than placebo, sulphasalazine or corticosteroid enemas.

In a single centre, single blinded, randomised, controlled trial, Pica 2013 included UC patients with left-sided mild to moderately ac- tive disease and randomised them to treatment with beclometha- sone dipropionate (BDP) 10 mg (n=30) once daily or 5-ASA enema (n= 32) for 8 weeks. All patients received a stable dose of 5- ASA 2.4 g/day. The primary outcome of remission was obtained in 42.9% of BDP treated patients compared with 63.9% of patients treated with 5-ASA enema, a difference that was not statistically significant. Patients treated with BDP had a mild suppression of morning cortisol concentrations. The authors concluded that BDP could be considered in patients with mild to moderately active left-sided UC who were non compliant to 5-ASA enemas.

In a study by Raj 2014 patients with moderately active UC were included in this open label, single centre, randomised controlled trial and treated with Mesalazine 800 mg two tablets three times daily (n=29) or prednisolone in tapering dose (n=25) for 6 weeks. Primary outcomes were clinical remission (Mayo score < 2), clinical response (decrease in Mayo score of 3 points) and endoscopic remission (sigmoidoscopy sub score < 1). Secondary outcome was changes in calprotectine concentrations. At week 6, 48% of patients treated with corticosteroids were in clinical remission as op- posed to 28% in the 5-ASA group (NS), while respectively 92% vs 89.7% showed a clinical response (NS). No statistically significant difference in endoscopic remission rates was observed between patients treated with corticosteroids or 5-ASA (respectively 68% and 65.5%). The authors concluded that there was no

difference in efficacy between Mesalazine and prednisolone for the treatment of acute flares of moderately active UC. This study was only published as an abstract. In a single centre, open label randomised controlled trial con- ducted in a pediatric population of newly diagnosed ulcerative colitis patients with left-sided colitis or pancolitis,

Romano 2010 randomised patients to treatment with 5-ASA (80 mg/kg; n = 15) or beclomethasone dipropionate (BDP, 5 mg/kg, n = 15) for a period of 8 weeks. Patients with extraintestinal manifestations, systemic complication or very distal disease (last 12-15 cm) were excluded from this study. Primary outcomes were clinical remission (PUCAI < 10) at week 4 and 12 and endoscopic remission (Barron 0-1) at week 12, secondary outcomes were histological remission. At week 4, 80% of patients treated with BDP achieved clinical remission as opposed to 33% in the 5-ASA group. Patients assigned to BDP had lower disease activity at both 8 ($P < 0.003$) and at 12 weeks ($P < 0.015$) than those who received 5-ASA. In 73% of BDP-treated patients colonoscopy showed endoscopic re- mission compared with 27% of in the 5-ASA group ($P < 0.025$). The authors concluded that BDP induced remission (clinical and endoscopic) significantly faster and more effectively than 5-ASA in pediatric mild-to-moderate UC.

Lastly, Sandborn 2012 compared two oral budesonide MMX dos- ing regimens to oral Mesalazine or placebo and was described earlier.

STUDIES COMPARING DIFFERENT DOSING REGI- MENS OF ORAL CORTICOSTEROIDS

Baron 1962 conducted a randomised, single-center, open label trial allocating patients with mild to moderate severe UC who failed first line treatment or prednisolone in a dose less than 20 mg a day to either low dose (20 mg) (n = 20) or high dose prednisolone (40 or 60 mg) (both n = 20) for 5 weeks. Patients with disease confined to the rectum or those who improved spontaneously were excluded. Outcomes were clinical and endoscopic remission or clinical and endoscopic response after five weeks of treatment. At the end of the study, patients receiving higher doses of corticosteroids (40 or 60 mg) were twice as likely to obtain remission as those treated with the low dose of corticosteroids (65% with in the high dose vs 30% in the low dose group). The authors concluded that high dose corticosteroids were more effective in inducing remission in mild to moderate severe UC than low dose corticosteroids.

Powell-Tuck 1978 conducted an open label, single blinded, randomised controlled trial in which UC patients with active proctocolitis were allocated to prednisolone given as a single dose (40 mg, n = 23)

or a divided day dose of prednisolone (10 mg 4 times daily, n = 22) for 4 weeks. The primary outcome was clinical re- mission (defined as a symptom score of 0), secondary outcomes were clinical response (defined as any improvement in symptoms) and steroid-induced side effects. Clinical remission was obtained by 23% in the divided dose group as opposed to 13% in the single dose group, while respectively 54% and 63% of patients had a clinical response after 4 weeks of treatment. These differences were not statistically significant. Side-effects did not differ significantly between groups. The authors concluded that prednisolone 40 mg as a single dose is the preferred treatment for active UC proctocolitis.

Additionally, studies by Sandborn 2012 and Travis 2014 respectively compared two oral budesonide MMX dosing regimens to oral Mesalazine or placebo and different oral budesonide MMX dosing regimens to placebo. Both were described earlier in the text.

STUDIES COMPARING ORAL CORTICOSTEROIDS TO TOPICAL FORMULATIONS (ENEMAS) In a single centre, open label randomised controlled trial Truelove 1960b included UC patients with mild to moderately severe dis- ease and allocated them to treated with local corticosteroid ene- mas (n=40), oral prednisolone 20 mg (n=40) or combined corticosteroid enema and oral prednisolone (n=40) for a duration of 2 weeks. The primary outcome was clinical remission defined as the absence of any symptoms and a decisive improvement on sigmoidoscopy. After two weeks of treatment 72.5% of patients treated with corticosteroid enemas were in clinical remission compared with 35% in the oral corticosteroid group and 85% in the combined treatment group. The authors concluded that combining oral and local corticosteroids is the most effective treatment for mild to moderately severe flares of UC.

Excluded studies

Fifty-one of the initially selected articles were not included in this review. Reasons for exclusion can be found in the “characteristics of excluded studies” table.

Risk of bias in included studies

A summary of the risk of bias is provided in Figure 2. Four included studies were considered of overall high methodological quality (Gross 2011; Rhodes 2008; Sandborn 2012; Travis 2014). All other studies had one or more methodological concerns which are discussed in detail in the sections below.

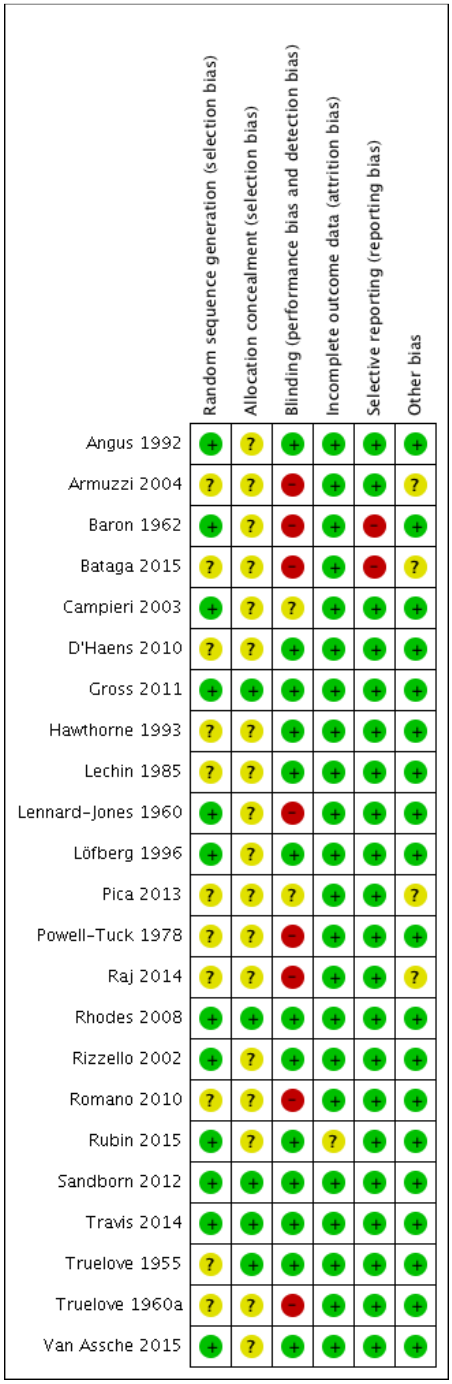

Figure 2. Risk of bias summary: review authors' judgements about each risk of bias item for each included study.

Allocation. In the study by Gross 2011 randomisation was based on a computer-generated list with randomly permuted blocks, with allocation only known to an externally contracted firm who was not involved in the study conduct resulting in a low risk of selection bias. In Rhodes 2008 randomisation was also computer-based and handled by the statistician, while allocation to treatment was performed by the hospital pharmacy based on the randomisation code. Both in Travis2014 and Sandborn 2012 risk of selectionbias was considered low because of a computer-generated randomisation with both using an external contractor with an interactive voice response system for allocation of treatment. Campieri 2003 also used a computer-based randomisation sequence.

In most other studies either the method of randomisation was not clearly described (Truelove 1955), the mode of allocation concealment was absent (Angus 1992; Baron 1962; Lennard-Jones 1960; Löfberg 1996; Rizzello 2002; Rubin 2015; Van Assche 2015) or both were missing (Armuzzi 2004; Bataga 2015; D'Haens 2010; Hawthorne 1993; Lechin 1985; Pica 2013; Powell-Tuck 1978; Raj 2014; Romano 2010; Truelove 1960a), resulting in an unclear risk of selection bias.

Blinding. Most of the studies included had a double blinded design with patients, treating physicians and outcome assessors blinded to the treatment allocation (Angus 1992; D'Haens 2010; Hawthorne 1993; Lechin 1985; Rhodes 2008; Rizzello 2002; Rubin 2015; Truelove 1955; Van Assche 2015) of which Angus 1992; D'Haens 2010; Lechin 1985; Rizzello 2002; Truelove 1955 and Van Assche 2015 clearly described using a matched control. Gross 2011; Löfberg 1996; Sandborn 2012 and Travis 2014 used a double dummy design. The risk of performance and detection bias in all of the described studies were considered to be low.

Campieri 2003; Pica 2013 and Powell-Tuck 1978 used a single blinded design with only treating physicians/outcome assessors blinded to the treatment allocation, but not the patients. Risk of bias in these studies was assessed as unclear/intermediate.

Several studies used an open label, unblinded study design and were determined to be at a high risk of performance and detection bias (Armuzzi 2004; Baron 1962; Bataga 2015; Lennard-Jones 1960; Raj 2014; Romano 2010; Truelove 1960a).

Incomplete outcome data. No evidence of incomplete outcome data reporting was found in the included studies. All studies had similar drop-outs rates in treated and control patients and reasons for drop-outs did not differ between groups.

Selective reporting. No evidence of selective reporting was found in most of the included studies. In both the studies of Baron 1962 and Bataga 2015 there was no reporting of the endoscopic data although this out- come was specified as the primary outcome. These studies were considered high risk for reporting bias.

Other potential sources of bias. Armuzzi 2004; Bataga 2015; Pica 2013 and Raj 2014 were rated as unclear for other sources of bias because these studies were only published in abstract from (no full publication available). The other studies appeared to be free of other sources of bias and were rated as low risk for this item.

Effects of interventions

See: **Summary of findings for the main comparison** Oral Corticosteroids compared to Placebo for Ulcerative colitis; **Summary of findings 2** Systemic Corticosteroids compared to Locally active corticosteroids for Ulcerative Colitis; **Summary of findings 3** Oral corticosteroids compared to 5ASA for Ulcervative Colitis; **Summary of findings 4** High dose corticosteroids compared to low dose corticosteroids for inducing remission in UC

ORAL CORTICOSTEROIDS VERSUS PLACEBO

Clinical remission Seven studies involving 1657 patients reported on clinical remission as an outcome (Rizzello 2002; D'Haens 2010; Rubin 2015; Sandborn 2012; Travis 2014, Truelove 1955; Angus 1992). The pooled analysis showed a statistically significant difference in clinical remission rates between corticosteroids and placebo. Twenty- eight per cent (281/1010) of patients in the corticosteroid group achieved clinical remission compared to 12% (77/647) of patients in the placebo group (RR 2.10, 95% CI 1.41 to 3.14). Statistical heterogeneity was moderate (I^2= 59%) (Figure 3). A GRADE analysis indicated that the overall quality of the evidence for the primary outcome (clinical remission) was low due to the important heterogeneity in the study results. Additionally, four of six studies in the pooled analysis were rated as unclear risk of bias for allocation concealment (3 studies) or random sequence generation (1 study). (See Summary of findings for the main comparison).

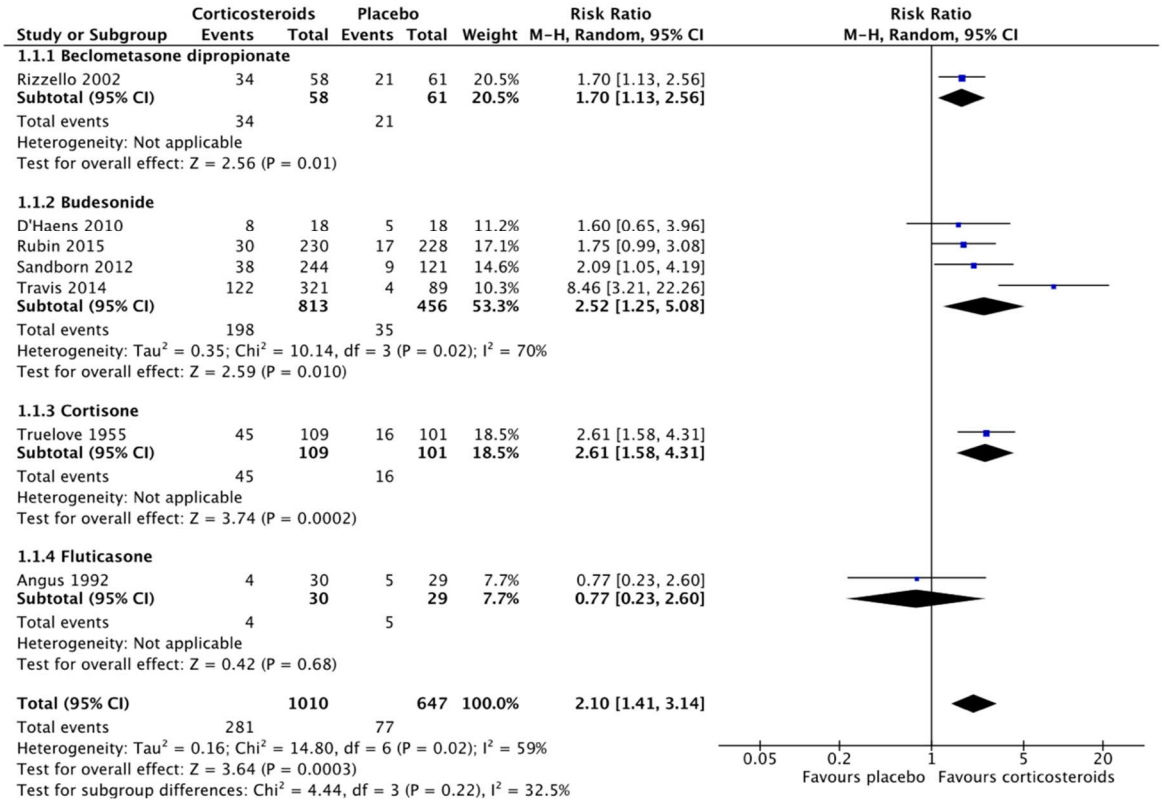

Figure 3 – Forrest plot comparing oral corticosteroids to placebo for inducing clinical remission

Clinical response Clinical response was reported on by five studies treating a total of 1163 patients (Angus 1992; Rizzello 2002; Sandborn 2012; Travis 2014; Truelove 1955). Pooled analysis showed a statistically significant higher response rate in patients treated with corticosteroids versus placebo. Thirty-seven percent (282/762) of patients in the corticosteroid treated group showed a clinical response after treatment versus 27% (108/401) in the placebo group. (RR 1.25, 95% CI 1.03-1.51). Statistical heterogeneity was low (I^2 = 0%). A GRADE analysis showed overall quality of the evidence to be moderate due to the fact that 3 of the 5 studies in the pooled analysis were rated as unclear risk of bias for random sequence generation (1 study) or allocation concealment (2 studies).

Endoscopic remission Five trials (n = 1013 patients) reported treatment outcomes in terms of endoscopic remission (Angus 1992; Rizzello 2002; Rubin 2015; Travis 2014; Truelove 1955). The pooled analysis using a random-effects model showed a statistically significant difference in endoscopic remission rates between corticosteroids and placebo. Twenty-one percent (106/509) of patients in the corticosteroid group achieved endoscopic remission compared to 11% of placebo patients (54/504) (RR 2.01, 95% CI 1.25 to 3.22) (Figure 4). Statistical heterogeneity was moderate (I^2= 45%).

A GRADE analysis indicated that the overall quality of the evidence for this outcome (endoscopic remission) was very low due to 1) sparse data (161 events), 2) 4/5 studies had an unclear risk of bias for either allocation concealment (2 studies) or random sequence generation (2 studies), 3) a moderate degree of heterogeneity (I^2= 45%) (See Summary of findings for the main comparison).

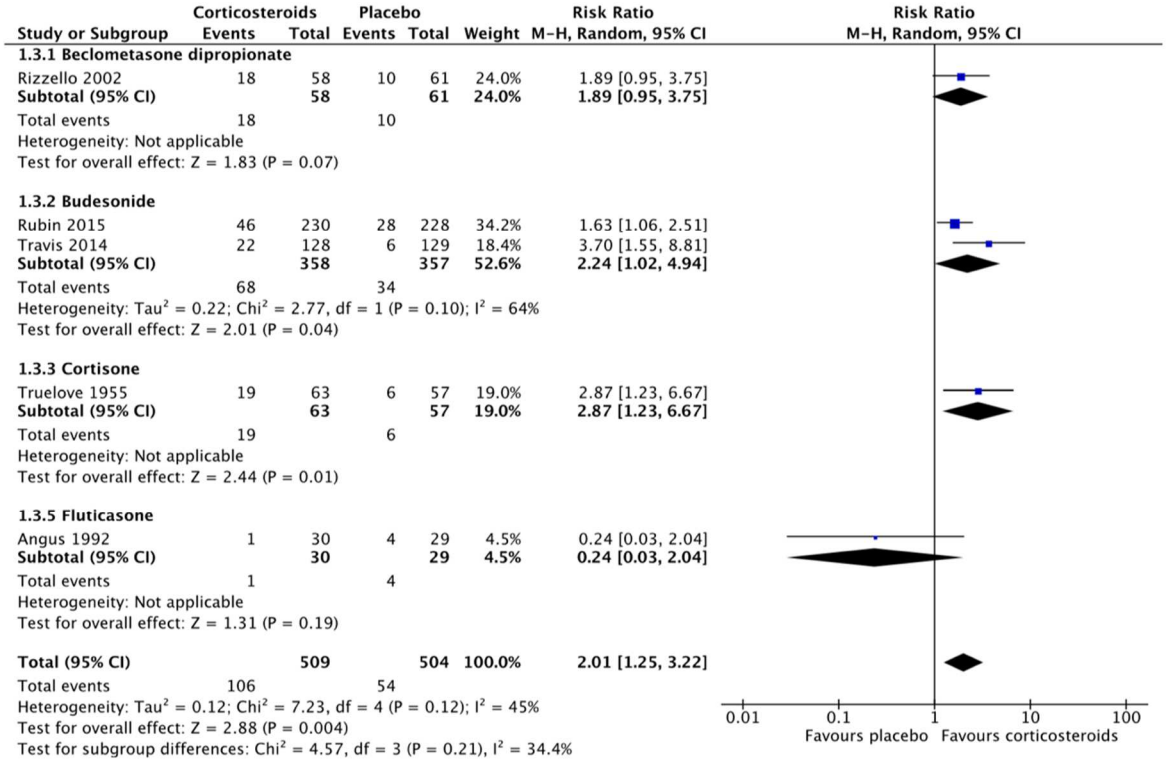

Figure 4 – Forrest plot comparing oral corticosteroids to placebo for inducing endoscopic remission

Endoscopic response Four studies (n = 954) reported treatment outcomes in terms of endoscopic improvement (Angus 1992; Sandborn 2012; Travis 2014; Truelove 1955). The pooled analysis showed a trend to- wards endoscopic improvement rates in the corticosteroid group compared to a placebo (P=.07). Thirty-eight per cent (252/658) of patients in the corticosteroid group achieved endoscopic improvement compared to 34% (100/296) of placebo patients (RR 1.19, 95% CI 0.98 to 1.44). Statistical heterogeneity was low (I^2 = 8%). A GRADE analysis indicated that the overall quality of the evidence for this outcome (endoscopic improvement) was considered as moderate due to the high risk of bias (blinding) in one of the four studies (See Summary of findings for the main comparison).

Histological remission Four studies (n=1391) reported on histologic remission as a treatment outcome (Angus 1992; Rubin 2015; Sandborn 2012; Travis 2014). The pooled analysis showed a statistically significant difference in histological remission rates between corticosteroids and placebo. Thirteen

percent (119/884) of patients in the corticosteroid group achieved histological remission compared to 11% (56/507) in the placebo group (RR 1.51 95% CI 1.12-2.04) (Figure 5). Statistical heterogeneity was low (I^2 = 19%). A GRADE analysis indicated that the overall quality of the evidence for this outcome (endoscopic improvement) was low due sparse data (175 events) and the fact that two of the four included studies had an unclear risk of bias for allocation concealment (See Summary of findings for the main comparison).

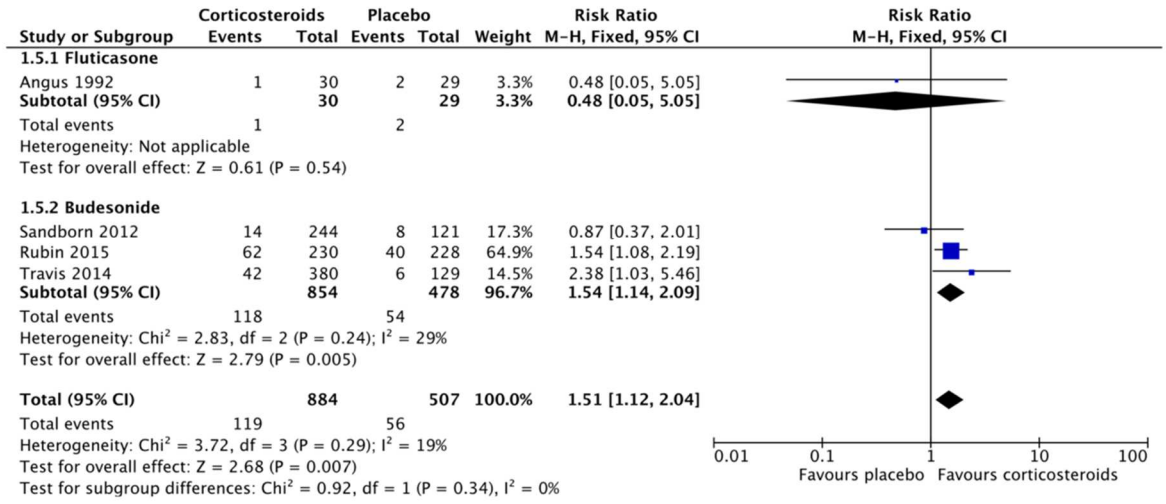

Figure 5 – Forrest plot comparing oral corticosteroids to placebo for inducing histological remission

Histological response Two trials (n = 257 patients) reported treatment outcomes in terms of histological improvement (Angus 1992; Travis 2014). The pooled analysis showed no statistically significant difference in histological improvement rates between corticosteroids and placebo. Twenty-one percent of patients (29/139) in the corticosteroids group achieved histological improvement compared to 13% (15/118) of placebo patients (RR 1.71, 95% CI 0.98 to 3.00). Statistical heterogeneity was low (I^2= 40%). A GRADE analysis indicated that the overall quality of the evidence for this outcome (histological improvement) was low due very sparse data and the low number of studies involved (44 events; See Summary of findings for the main comparison).

Ulcerative Colitis Disease Activity Index (UCDAI) Two trials (n=155 patients) reported on UCDAI scores as an outcome (D'Haens 2010; Rizzello 2002). The pooled analysis showed a statistically significant difference in UCDAI scores favoring corticosteroids over placebo (MD 0.88, 95% CI 0.21 to 1.54). Heterogeneity was low for this comparison (I^2 = 11%).

Treatment failure requiring additional treatment One trial (n = 210 patients) reported treatment

outcomes in terms of treatment failure requiring additional treatment (Truelove 1955). There was no statistically significant difference in treatment failure requiring additional treatment between corticosteroids and placebo. Eight percent (9/109) of patients in the corticosteroid group failed treatment and required additional treatment compared to 14% (14/101) of patients who received placebo (RR 0.60, 95% CI 0.27 to 1.32).

Adverse events Six trials (n = 1731 patients) reported on the proportion of patients who had an adverse event (Angus 1992; Rizzello 2002; Rubin 2015; Sandborn 2012; Travis 2014; Truelove 1955). Fortyfour percent (465/1062) of patients in the corticosteroid group had an adverse event compared to 33% (221/669) of patients in the placebo group (RR 1.09, 95% CI 0.87 to 1.37), but this was not statistically significant (P=0.46) (Figure 6). Statistical heterogeneity was moderate (I^2= 56%). A GRADE analysis indicated that the over- all quality of the evidence for this outcome (adverse events) was moderate due to the considerable heterogeneity in the data. (See Summary of findings for the main comparison).

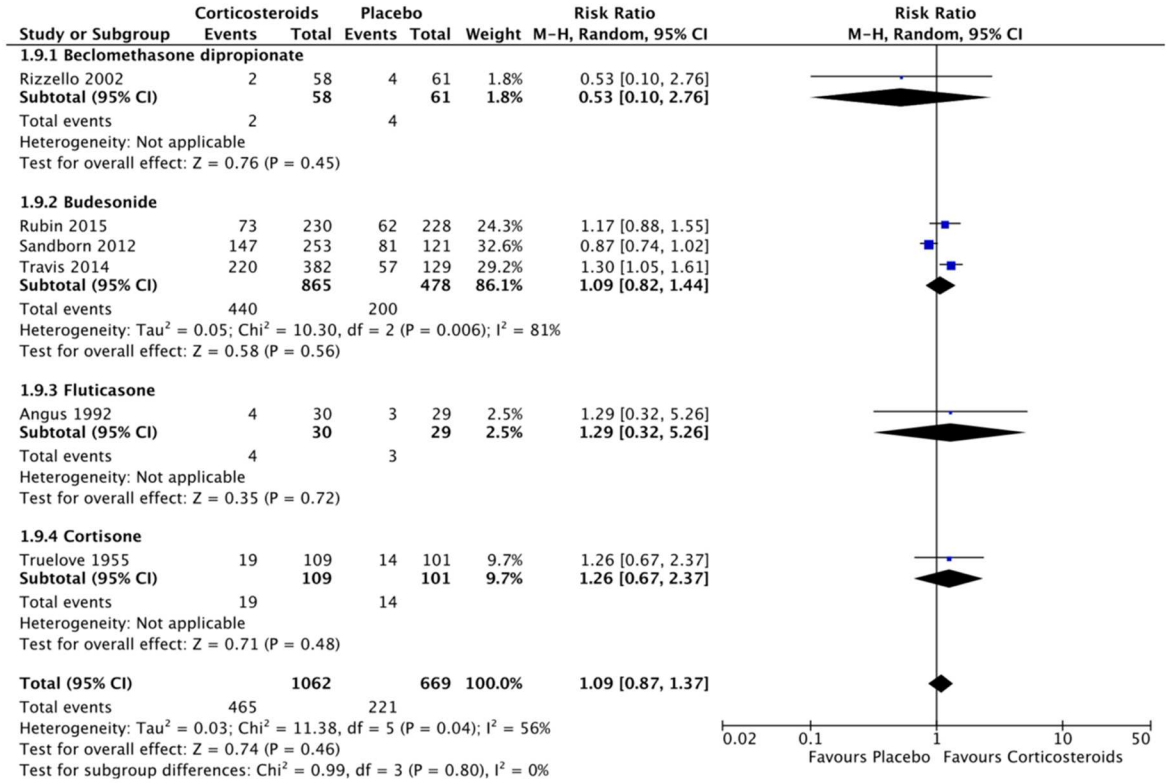

Figure 6 – Forest plot comparing oral corticosteroids to placebo in inducing adverse events

Withdrawal due to adverse events Six trials (n = 1641 patients) reported on withdrawal due to adverse events (Angus 1992; Rizzello 2002; Rubin 2015; Sandborn 2012; Travis 2014; Truelove 1955). The pooled analysis showed no statistically significant difference in the proportion of patients who withdrew due to an adverse event between the corticosteroid group and placebo. Twelve percent in the corticosteroid group (123/1016) versus 9% in the placebo group (56/625) withdrew due to adverse events and reasons for withdrawal were similar be- tween groups. (RR 1.00, 95% CI 0.74 to 1.34). Heterogeneity was low for this comparison (I^2 = 26%). One study (Truelove 1955) commented on withdrawal rates due to treatment failure with 9/109 (8.3%) patients in the corticosteroid group vs 14/101 (13.8%) in the placebo group required additional treatment.

LOCALLY ACTIVE CORTICOSTEROIDS VS SYSTEMIC CORTICOSTEROIDS

Clinical remission Three studies involving 669 patients reported on clinical remission as an outcome (Hawthorne 1993; Rhodes 2008; Van Assche 2015). The pooled analysis showed no significant difference in clinical remission rates between local and systemic corticosteroids. Twenty-six percent (94/358) of patients in the locally active corticosteroid group achieved clinical remission compared to 29% (90/ 311) of patients in the systemic corticosteroid group (RR 0.84, 95% CI 0.65 to 1.08) (Figure 7). There was no statistical heterogeneity (I^2= 0%). A GRADE analysis indicated that the overall quality of the evidence for the primary outcome (clinical remission) was low due to 1) sparse number of data (184 events) 2) the fact that 2 of the three studies had an unclear risk of bias for allocation concealment, while one study had an unclear risk of bias for random sequence generation (See Summary of findings 2).

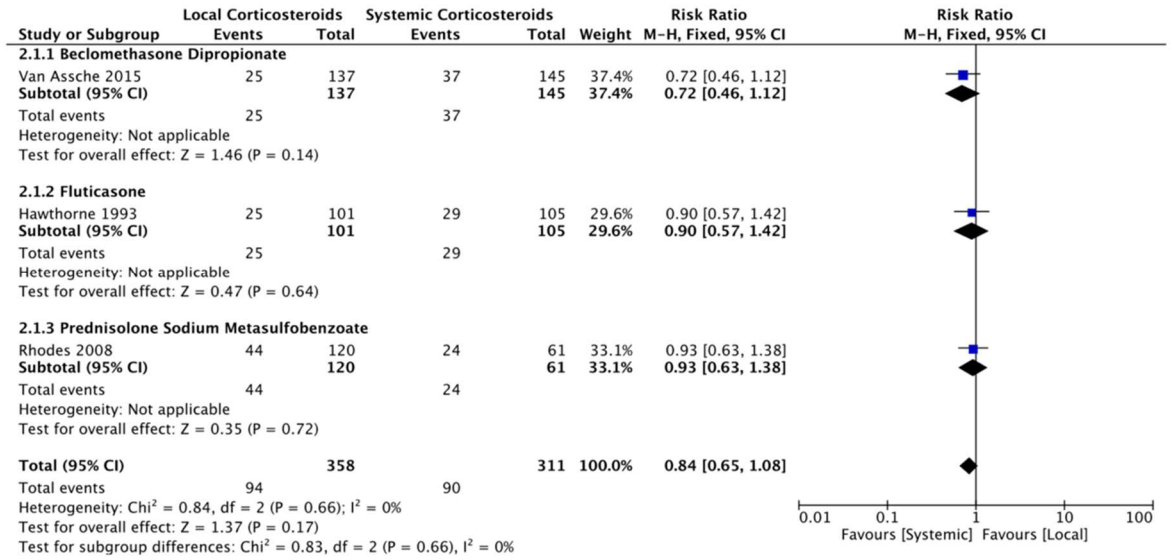

Figure 7 – Forrest plot comparing systemic vs locally active corticosteroids for inducing clinical remission

Clinical response Two studies that evaluated 488 patients investigated clinical response as an outcome (Hawthorne 1993; Van Assche 2015). Pooled analysis showed no statistically significant difference in clinical response rates between locally active and systemic corticosteroids. Fifty percent of patients treated with locally active corticosteroids (120/238) versus 55% of patients treated with systemic corticosteroids (138/250) achieved a clinical response (RR 0.82, 95% CI 0.57-1.18). Statistical heterogeneity was low (I^2 = 5.5%). Overall quality of evidence was considered to be low in a GRADE analysis due to 1) low number of events (N=258) and 2) all of the included studies had an unclear risk of bias for allocation concealment, while one of the two additionally had an unclear risk of bias for random sequence generation (See Summary of findings 2).

Endoscopic remission Four studies that included a total of 735 patients reported on endoscopic remission (Hawthorne 1993; Löfberg 1996; Rhodes 2008; Van Assche 2015,). There was a statistical trend (P=0.07) towards increased endoscopic remission in patients treated with systemic corticosteroids. Thirty-seven percent of patients treated with locally active corticosteroids were in endoscopic remission at the end of the trial periods (132/356) versus 41% (132/324) in the systemic corticosteroid treated group (RR 0.78, 95%CI 0.60, 1.02) (Figure 8). Statistical heterogeneity was moderate (I^2 = 52%). Overall quality of evidence by GRADE analysis was considered low due to 1) considerable heterogeneity (I^2 = 52%) in the data and 2) the fact that three of the four studies had an

unclear risk of bias for allocation concealment and 1 study was additionally rated as unclear risk of bias for random sequence generation (See Summary of findings 2).

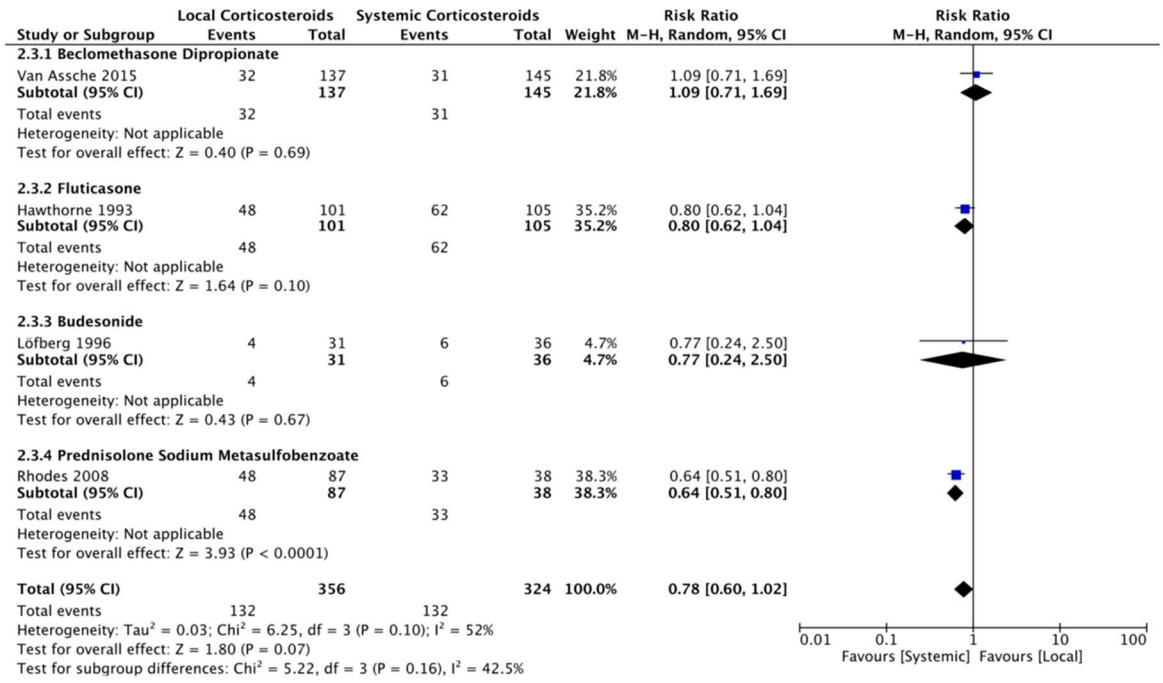

Figure 8 – Forrest plot comparing systatmic to locally active corticosteroids in inducing endoscopic remission

Endoscopic response Endoscopic response was reported on by only 1 study (Löfberg 1996) which found no statistically significant difference between patients treated with locally active or systemic corticosteroids (RR 0.98 95% CI 0.70-1.35). Overall quality of evidence by GRADE analysis was considered very low because of 1) low number of events (N=46); 2) the fact that the included study had unclear risk of bias for allocation concealment and 3) the fact that only one study examined this endpoint.

Histological remission Only 1 study (Löfberg 1996) provided results on histological remission. The authors found no statistically significant difference in histological remission rates between patients treated with locally active or systemic corticosteroids (RR 0.50 95% CI 0.14-1.82) (Figure 8). GRADE analysis for the quality of evidence was very low because 1) a very low number of observation (N=9); 2) the study had an unclear risk of bias for allocation concealment and 3) the fact that only one study examined this endpoint.

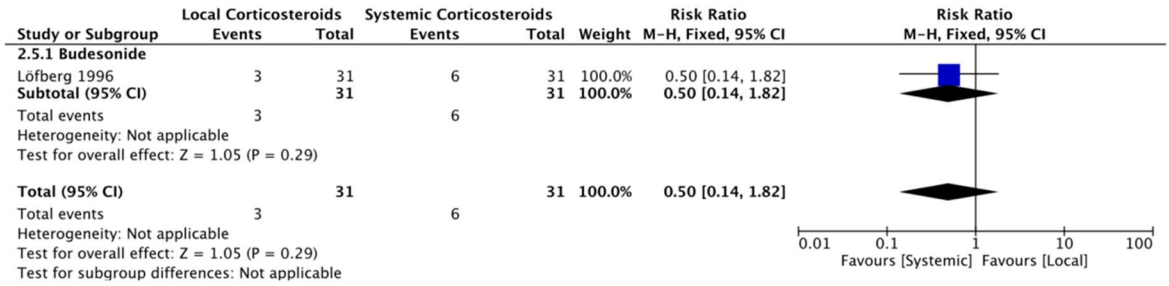

Figure 8 – Forrest plot comparing systemic to locally active corticosteroids in inducing histological remission

Histological response Two trials including a total of 273 patients reported on histological response rates (Löfberg 1996; Hawthorne 1993). There was a statistically significant difference favoring systemic corticosteroids. Twenty-three percent (30/132) of patients treated with locally active corticosteroids achieved a histological response at the end of the study period versus 45% (63/141) of patients with systemic treatment (RR 0.52 95% CI 0.36-0.74). There was no statistical heterogeneity (I^2 = 0%). However, quality of evidence was judged to be low according to a GRADE analysis due to the sparse number of events (N=93) and the fact that both studies had an unclear risk of bias for allocation concealment, while one study (Hawthorne 1993) had an additional unclear risk of bias for random sequence generation (Summary of findings 2).

Adverse events Adverse events were reported in three trials with a total of 668 patients (Hawthorne 1993; Rhodes 2008; Van Assche 2015). There was a statistically significant difference favoring locally active corticosteroids in the occurrence of adverse events. In patients treated with locally active corticosteroids 23% (82/358) reported at least one adverse event versus 23% (71/310) in the patients treated with systemic corticosteroids (RR 0.67 95% CI 0.54-0.82)). There was moderate statistical heterogeneity (I^2 = 43%) (Figure 9). A GRADE analysis found the evidence to be of low quality due to the sparse number of events (N=153) and the fact that 2 out of 3 studies included in the analysis were scored as having an unclear risk of bias for al- location concealment. Additionally, one study (Hawthorne 1993) had an unclear risk of bias for random sequence generation (See Summary of findings 2).

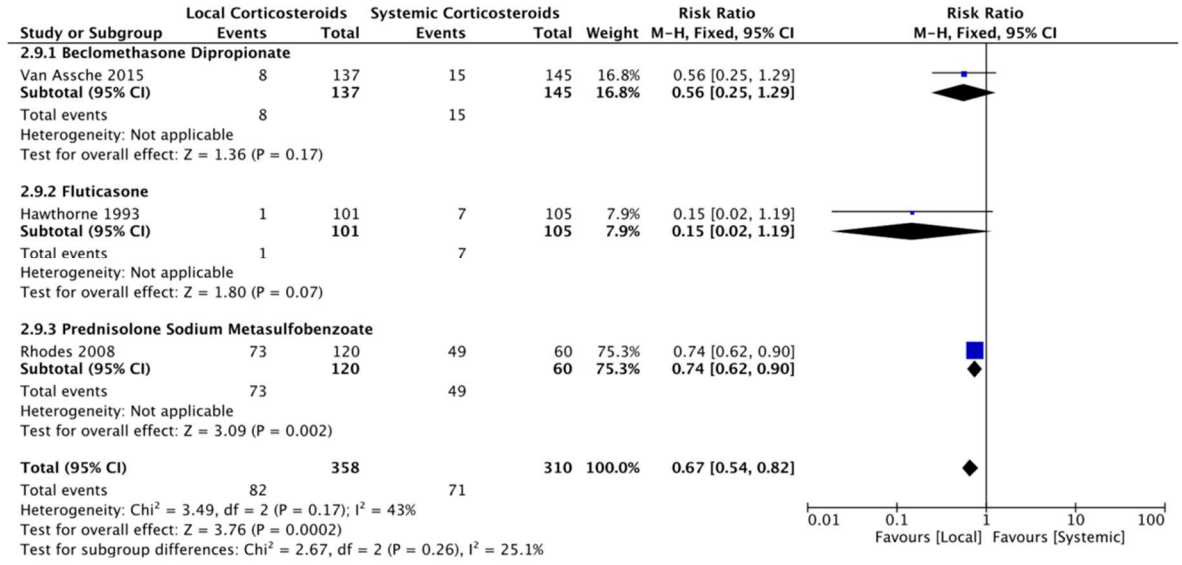

Figure 9 – Forrest plot comparing systemic to locally active corticosteroids for inducing adverse events

Withdrawal due to adverse events Four trials reported the withdrawal rate due to adverse events in a total of 735 patients (Hawthorne 1993; Löfberg 1996; Rhodes 2008; Van Assche 2015). There was no statistical significant difference in withdrawal rate between groups (RR 0.62 95% CI 0.30- 1.27). Three percent of patients treated with local corticosteroids (13/389) in these trials withdrew because of adverse events vs 5% in the systemic corticosteroid group (16/346). There was no statistical heterogeneity (I^2 =0%).

ORAL CORTICOSTEROIDS VS 5-ASA

Clinical remission Seven studies involving 1074 patients reported on clinical remission as an outcome (Campieri 2003; Gross 2011; Lennard-Jones 1960; Pica 2013; Raj 2014; Romano 2010; Sandborn 2012). The pooled analysis showed no significant difference in clinical remission rates between corticosteroids and 5-ASA. Thirty-five percent (213/601) of patients in the corticosteroid group achieved clinical remission compared to 42% (201/473) of patients in the 5-ASA group (RR 1.09, 95% CI 0.81 to 1.45) (Figure 10). The statistical heterogeneity was high (I^2= 67%). A GRADE analysis indicated that the overall quality of the evidence for the primary outcome (clinical remission) was very low due to a high inconsistency in the data (I^2= 67%) and the fact that two of the five studies included were graded as of high risk of basis due to problems with blinding (See Summary of findings 3).

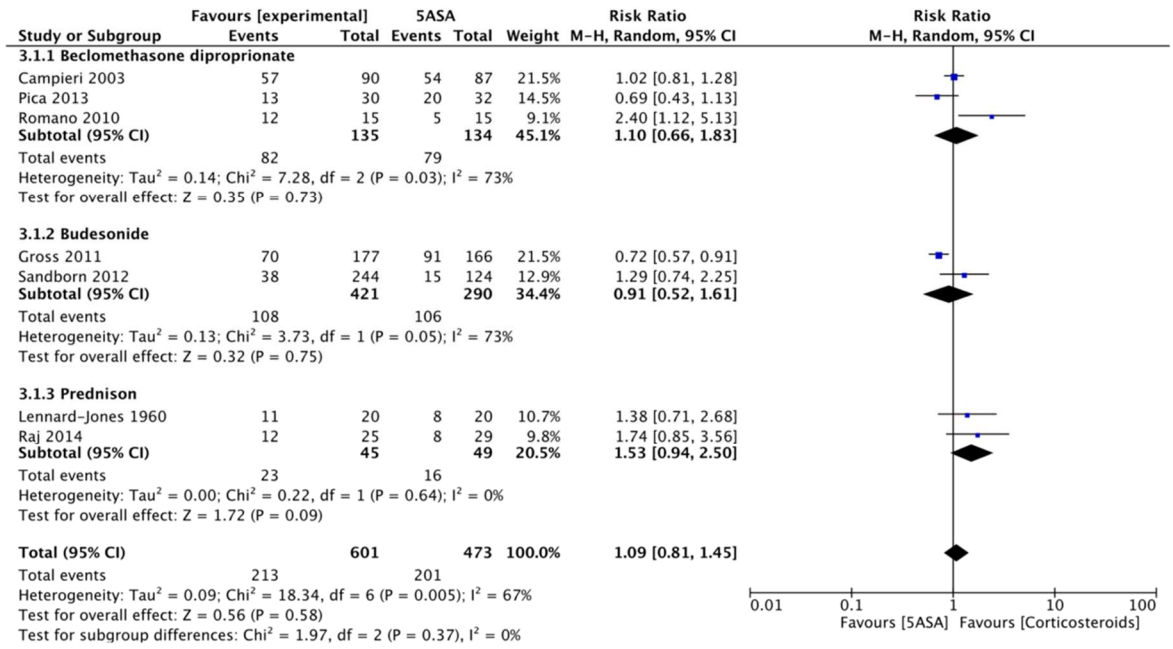

Figure 10 – Forrest plot comparing oral corticosteroids to 5-ASA for inducing clinical remission

Clinical response

Five studies treating 669 patients investigated clinical response as an outcome (Campieri 2003; Lechin 1985; Lennard-Jones 1960; Raj 2014; Sandborn 2012). Pooled analysis showed no statistically significant difference in clinical response rates between corticosteroids (33%, 130/394) and 5-ASA (33%, 90/275) (RR 1.04, 95% CI 0.85-1.27). Statistical heterogeneity was low (I^2 = 0%). Overall quality of evidence was considered to be low in a GRADE analysis due to a low number of events (N=220) and the fact that 2 out of 5 studies included in the analysis were rated as of high bias risk (blinding) (See Summary of findings 3).

Endoscopic remission

Three studies including a total of 427 patients reported on endoscopic remission (Gross 2011; Raj 2014; Romano 2010). There was no statistically significant difference in achieving endoscopic remission between both groups. Fifty-three percent of patients (116/217) treated with local corticosteroids were in endoscopic re- mission at the end of the trial period versus 61% (128/210) in the systemic corticosteroid treated group (RR 0.88 95% CI 0.75- 1.04) (Figure 11). Statistical heterogeneity was high (I^2 = 76%). Overall quality of evidence via GRADE analysis was considered very low due to the high inconsistency in the data (I^2 = 76%) and overall low quality of studies (e.g. 2/3

studies had a high risk of bias for blinding) (See Summary of findings 3).

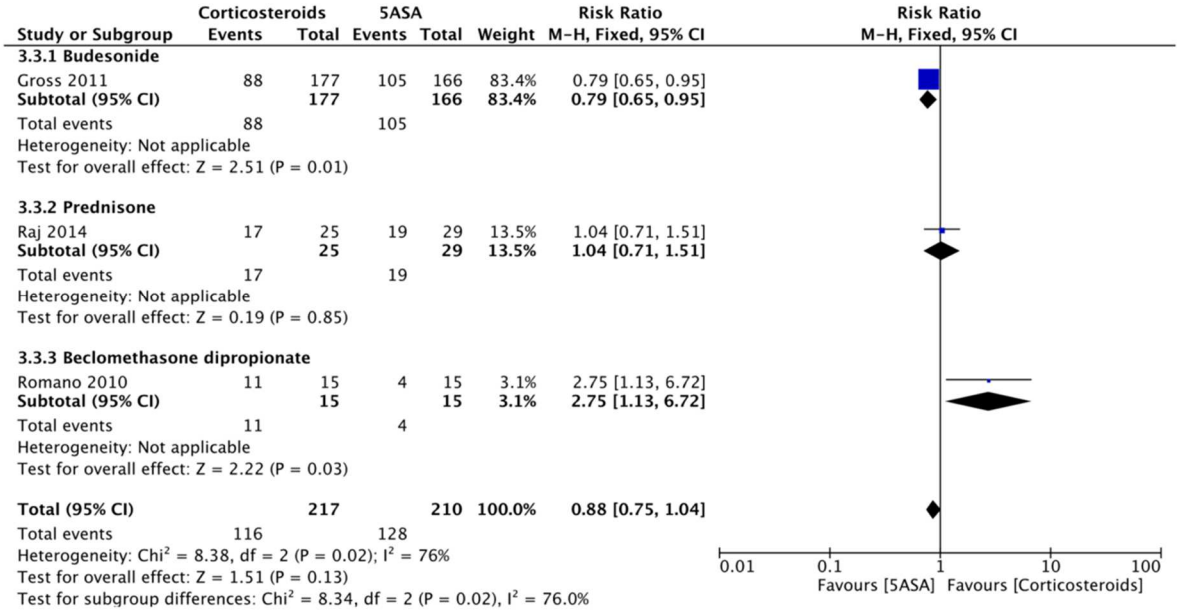

Figure 11 – Forrest plot comparing 5-ASA to oral corticosteroids for inducing endoscopic remission

Endoscopic response Endoscopic response was reported on by two studies (Gross 2011; Sandborn 2012) in 711 patients which found no statistically significant difference between patients treated with corticosteroids or 5-ASA (respectively 51% (216/421) in the corticosteroid group vs 61% (177/290) in the 5-ASA treated patients (RR 0.93 95% CI 0.82-1.06)). Statistical heterogeneity was high (I^2 = 79%) and the quality of evidence was considered low due to the very serious inconsistency in the data (See Summary of findings 3).

Histological remission Three studies (Gross 2011; Romano 2010; Sandborn 2012,) showed results on histological remission (n=741). Pooled analysis found a statistically significant difference in histological remission rates favouring patients treated with 5-ASA. Twenty percent of patients treated with corticosteroids (89/436) achieved histological remission versus 38% (115/305) in the 5-ASA group (RR 0.67 95% CI 0.54-0.83) (Figure 12). Statistical heterogeneity was high (I^2= 77%) and the evidence was rated as of very low quality due to the high inconsistency in the data and the low number of events (N=204) (See Summary of findings 3).

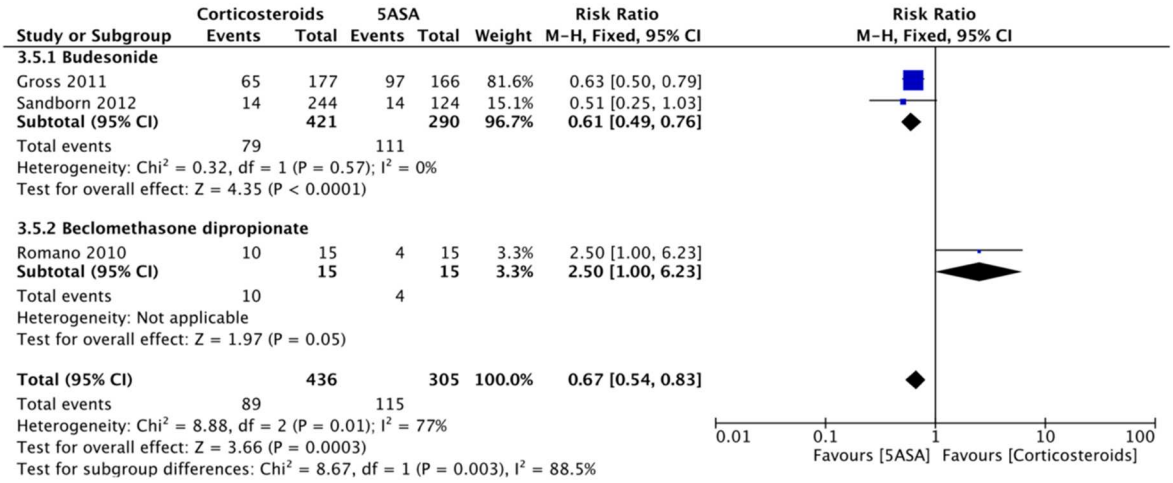

Figure 12 – Forrest plot comparing 5-ASA to oral corticosteroids for inducing histological remission

Histological response Only one study including a total of 343 patients reported on histological response rates (Gross 2011). There was a statistically significant difference favouring 5-ASA. Fifty-seven percent (101/ 177) of patients treated with corticosteroids achieved a histological response at the end of the study period versus 72% (120/166) of patients with 5-ASA (RR 0.79 95% CI 0.67-0.93). The quality of the evidence was graded as of overall moderate quality due to the sparse number of events (N=221) (See Summary of findings 3).

Ulcerative Colitis Disease Activity Index (UCDAI) Three trials (n = 550 patients) reported on UCDAI scores as an outcome (Campieri 2003; Gross 2011; Romano 2010). The pooled analysis showed a statistically significant difference in UC- DAI scores favouring 5-ASA treated patients (MD -0.72, 95% CI 1.44 to 0.00). Statistical heterogeneity was moderate for this comparison (I^2 = 67%).

Adverse events Adverse events were reported in four trials with a total of 959 patients (Campieri 2003; Gross 2011; Sandborn 2012; Lennard- Jones 1960). There was no statistically significant difference in the occurrence of adverse events. In patients treated with corticosteroids 43% (242/562) reported at least one adverse event versus 34% (135/397) in the patients treated with 5-ASA (RR 0.95 95% CI 0.70-1.29) (Figure 13). There was a moderate statistical heterogeneity (I^2 = 53%). A GRADE analysis found the evidence to be of low quality due to the moderate inconsistency of the data and the fact that one of the four studies included in the analysis was of high risk of basis for blinding (See Summary of findings 3).

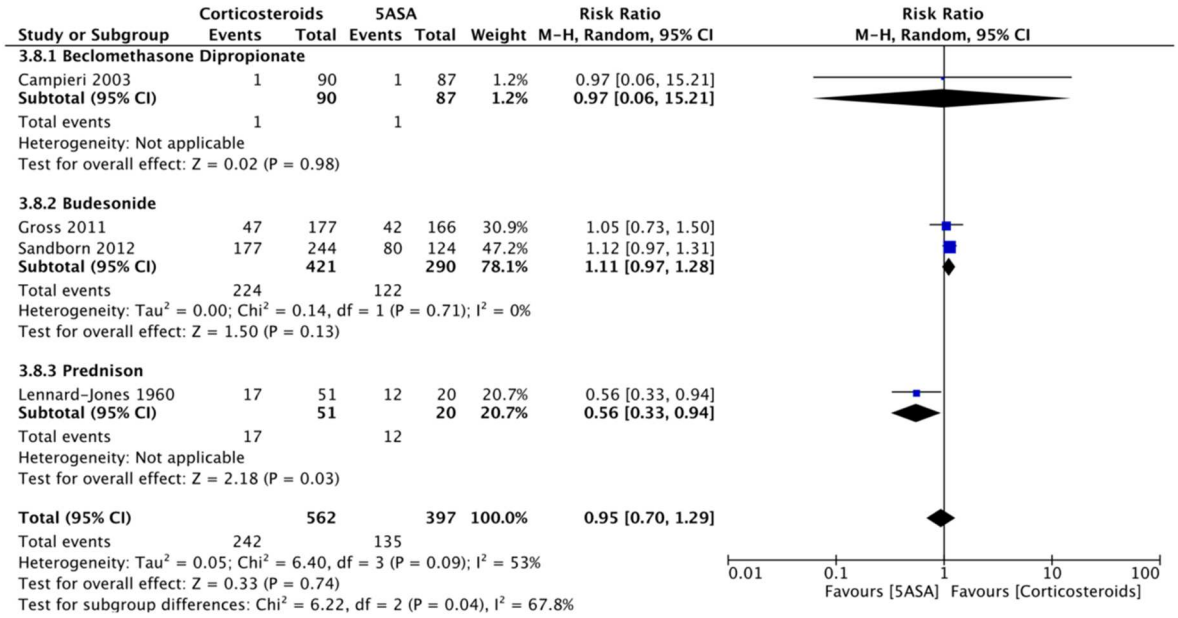

Figure 13 – Forrest plot comparing 5-ASA to oral corticosteroids for inducing adverse events

Withdrawal due to adverse events. Three trials reported the withdrawal rate due to adverse events in a total of 888 patients (Campieri 2003; Gross 2011; Sandborn 2012). There was no statistical significant difference in withdrawal rate between groups (RR 1.43 95% CI 0.90-2.29). Ten percent of patients treated with corticosteroids (50/511) in these trials withdrew because of adverse events vs 6% in the 5-ASA group (22/377). There was no statistical heterogeneity (I^2=0%).

HIGH DOSE CORTICOSTEROIDS VERSUS LOW DOSE CORTICOSTEROIDS

Clinical remission. Five studies involving 801 patients reported on clinical remission as an outcome (Baron 1962; Powell-Tuck 1978; Rhodes 2008; Sandborn2012; Travis2014). The pooled analysis showed a significant difference in clinical remission rates favouring higher dosed corticosteroid regimens. Twenty-nine percent (132/463) of patients who received high dose corticosteroids achieved clinical re- mission compared to 18% (61/338) of patients in the low dose group (RR 1.56, 95% CI 1.17 to 2.08). The statistical heterogeneity was high (I^2= 84%). Overall quality of the evidence was considered to be very low according to a GRADE analysis due to 1) high inconsistency in the data (I^2= 84%), 2) a low number of events (N=193) and 3) the fact that 2 of the five studies included in the analysis had a high risk of bias in regard to blinding.

Clinical response. Four studies that enrolled 680 patients investigated clinical response as an outcome

(Baron 1962; Powell-Tuck 1978; Sandborn 2012; Travis 2014). Pooled analysis showed a statistically significant difference in clinical response rates favouring higher dosed corticosteroids. Thirty-five percent of patients treated with higher dosed corticosteroids (142/401) achieved a clinical response as op- posed to 29% in the low dose group (80/279) (RR 1.27, 95% CI 1.01-1.60). Statistical heterogeneity was low (I^2 = 0%). A GRADE analysis showed the evidence to be of low quality due to 1) a low number of events (N=222) and 2) the fact that 2/4 studies had high risk of bias in regard to blinding.

Endoscopic remission. Only 1 study (Rhodes 2008) reported on endoscopic remission. The authors found no statistically significant difference in achieving endoscopic remission between high dose and low dose corticosteroids (respectively 22/37 (59%) and 26/40 (65%) (RR 0.91 95% CI 0.64-1.30). A GRADE analysis showed this evidence to be of low quality due to the low number of events (N=48) and the fact that only one study reported on the outcome.

Endoscopic response. Endoscopic response was reported on by two studies (Sandborn 2012; Travis 2014) in 576 patients which found a statistically significant difference in favour of high dose corticosteroids (40% (135/339) in the high dose group vs 30% (71/237) in the low dose (RR 1.36 95% CI 1.07-1.73)). Statistical heterogeneity was low (I^2 = 7%). The evidence was graded as of moderate quality (GRADE analysis) due to the low number of events (N=206).

Histological remission. Two studies (Sandborn 2012; Travis 2014) showed results on histological remission (n=576). There was no statistically significant difference in histological remission rates between both groups (11% (37/339) in the high dose group vs 8% (19/237) in the low dose group (RR 1.04 95% CI 0.37-2.96). Statistical heterogeneity was moderate (I^2= 64%). The quality of evidence was graded as of low quality due to the significant inconsistency in the data and the low number of events (N=58).

Histological response. None of the included studies reported on histological response.

Ulcerative Colitis Disease Activity Index (UCDAI). None of the included studies reported on UCDAI.

Adverse events. Adverse events were reported in five trials with a total of 801 patients (Baron 1962; Powell-Tuck 1978; Rhodes 2008; Sandborn 2012; Travis 2014). There was no statistically significant difference in the occurrence of adverse events. In patients treated with high dose corticosteroids 58% (270/463) reported at least one ad- verse event versus 60% (203/338) in the patients treated with low

dose corticosteroids (RR 0.96 95% CI 0.86-1.08)). There was no statistical heterogeneity (I^2 = 0%). Overall quality of the evidence was graded as of moderate quality due to the fact that 2 of the 5 included studies had a high risk of bias in regard to blinding.

Withdrawal due to adverse events. Four trials reported the withdrawal rate due to adverse events in a total of 756 patients (Baron 1962; Rhodes 2008; Sandborn 2012; Travis 2014). There was no statistical significant difference in withdrawal rate between groups (RR 0.82 95% CI 0.59 - 1.13). Fifteen percent of patients treated with high dose corticosteroids (67/440) in these trials withdrew because of adverse events vs 17% in the low dose group (53/316). There was no statistical heterogeneity (I^2 =0%).

ORAL CORTICOSTEROIDS VERSUS OTHER ACTIVE COMPARATORS. One study compared oral corticosteroids to TNF antagonists (Armuzzi 2004) (N=20). All patients in both groups were in clinical remission at the end of the study. Another study evaluated clinical remission rates in UC patients treated with oral corticosteroids or antibiotics (n=48) and found no difference between the groups (Bataga 2015).

ORAL CORTICOSTEROIDS VERSUS TOPICAL CORTI- COSTEROIDS (ENEMAS) One study compared oral corticosteroids to topical corticosteroids administrated by enema (n=80, Truelove 1960a). Thirty-five per- cent of patients treated with oral corticosteroids (14/40) reached clinical remission as opposed to 73% (29/40) in the patients treated with corticosteroid enemas (RR 0.48 95% CI 0.30-0.77).

DISCUSSION

Summary of main results

In accordance with current treatment guidelines, oral corticosteroids are recommended as second-line therapy for induction of remission in acute flares of UC in patients with mild to moderately active disease. (Magro 2017) First reports regarding the efficacy and safety of corticosteroids were published in the 1950's and since then they have they have come into widespread use for the treatment of patients who fail 5-ASA and in those with sufficiently high disease activity that 5-ASA is not an adequate first-line therapy. However, potentially serious systemic side-effects constrain use of systemic corticosteroids and clinicians must carefully consider the therapeutic index in a given patient. Although the development of locally active corticosteroids, including budesonide, beclomethasone dipropionate, fluticasone propionate and prednisolone metasulfobenzoate, that have a more localized action mechanism due to high first hepatic metabolism offers a safer option, questions

remain regarding their relative effectiveness to the systemic agents. In this systematic review and meta-analysis, we examined the efficacy and safety of both systemic and locally active oral corticosteroids for induction of remission in active UC.

We identified seven trials ((Angus 1992; D'Haens 2010; Rizzello 2002; Rubin 2015; Sandborn 2012; Travis 2014; Truelove 1955) that compared the use of oral corticosteroids to placebo for the induction of remission in UC. Oral corticosteroids were significantly more effective in achieving clinical remission and response compared to placebo. Contrary to general belief, treatment with oral corticosteroids did not only ameliorate symptoms but was also more effective in inducing both mucosal healing and histological remission in acute flares of UC compared to placebo. Although the occurrence of adverse events was higher in the oral corticosteroid group, the difference was not statistical significant. Failure to demonstrate significance may be due to the relative lack of reporting of safety events in the older studies. Similarly, although with-drawal due to adverse events were no more common in patients treated with corticosteroids than those who received placebo, this finding may reflect a trade-off between patients being withdrawn due to corticosteroid-related adverse events in the former group and those being withdrawn due to disease worsening in the latter. Of note, all but one study (Truelove 1955) included in this analysis examined the use of locally active corticosteroids vs placebo, which might also explain the lack of a difference in adverse event rate between groups.

systemic corticosteroids were compared to locally active agents in four studies (Hawthorne 1993; Löfberg 1996; Rhodes 2008; Van Assche 2015). There was no statistical difference between systemic and locally active corticosteroids in inducing clinical remission or response in patients with an acute flare of ulcerative colitis. Systemic corticosteroids showed a trend towards inducing endoscopic remission more frequently (41% vs 37%) and achieving a histological response as compared to locally active corticosteroids, but were associated with a significantly higher adverse event rate, although this did not result in a more frequent study withdrawal. We note that a Cochrane review that evaluated this question in Crohn's disease found similar results with patients treated with systemic corticosteroids achieving remission more frequently than patients treated with locally active corticosteroids (Rezaie 2015). Five studies compared higher dosed oral corticosteroids to a lower dose of the same agents (Baron 1962; Powell-Tuck 1978; Rhodes 2008; Sandborn 2012; Travis 2014) and found higher doses of corticosteroids to be more effective in inducing clinical remission and response. Patients treated with higher doses were also more likely to achieve an endoscopic response, but there was no difference in endoscopic or histological remission rates. One study compared oral corticosteroids to topical corticosteroids administered via enema (Truelove 1955) and found clinical remission rates to be significantly higher in the latter group.

A number of studies compared the efficacy of oral corticosteroids to other treatments. Eight studies compared the efficacy of oral corticosteroids to oral 5-ASA (Campieri 2003; Gross 2011; Lechin 1985; Lennard-Jones 1960; Pica 2013; Raj 2014; Romano 2010; Sandborn 2012). Combined analysis of these studies showed no differences between oral corticosteroids and 5-ASA for induction of clinical remission or response. Additionally, there were no differences in endoscopic remission or response between patients treated with either treatment. Patients treated with 5-ASA, how- ever, achieved histologic response and even remission significantly more frequently than patients treated with oral corticosteroids (respectively, 72% vs 57% for histological response, 38% vs 20% for histological remission). Disease activity scores were also significantly lower in patients treated with 5-ASA than those treated with corticosteroids. Numerically, the number of adverse events or withdrawal from the study due to adverse events was higher in the oral corticosteroid group compared to 5-ASA (respectively 9.7% vs 5.8%), although this was not significantly different. This finding is in keeping with the wealth of clinical experience that indicates 5-ASA is safer than oral corticosteroid therapy. Although the observed lack of difference in remission and endoscopy between these classes of drugs cannot be taken as proof of non-inferiority for 5-ASA, the superiority demonstrated for histopathology is a potentially important finding. All of these findings should be interpreted with caution due to low GRADE scores. Furthermore, we expect that these results are only to be generalized to the milder end of the disease spectrum based on the possibility that these patients would be more likely recruited into the studies.

The one study that compared oral corticosteroids to TNF antagonists (Armuzzi 2004) found no difference in clinical remission rates in severe UC. A second study (Bataga 2015) that compared oral corticosteroids to antibiotics found similar remission rates between the two groups. Results of these studies should be interpreted with caution due to the low number of patients included and important limitations in the study design (e.g. open label design).

Overall completeness and applicability of evidence

In general the results of this review are applicable to patients with acute flares of mild-to-moderate ulcerative colitis. This review makes use of twenty-three published randomised trials. Most of the included studies were multicenter trials conducted in countries where the burden of ulcerative colitis is greatest. Nine of these studies were large multicenter, blinded randomised controlled trials (Campieri 2003; Gross 2011; Hawthorne 1993; Rhodes 2008; Rizzello 2002; Rubin 2015; Sandborn 2012; Travis 2014; Truelove 1955), while (Angus 1992; D'Haens 2010; Lechin 1985; Lennard-Jones 1960; Löfberg 1996; Pica 2013, Powell-Tuck 1978) were pilot studies with relatively small numbers of patients. The remaining seven studies were open label, non blinded small studies (Armuzzi 2004;

Baron 1962; Bataga 2015; Lennard-Jones 1960; Raj 2014; Romano 2010; Truelove 1960a). The studies were conducted in adult patients with mild-to-moderate ulcerative colitis, with the majority of the patients having left-sided disease, only one study was performed in a paediatric population (Romano 2010). The overall findings of this review support the use of oral corticosteroids for inducing remission in active ulcerative colitis. Locally active corticosteroids (budesonide, beclomethasone dipropionate, fluticasone propionate and prednisolone metasulfobenzoate) are equally effective in inducing remission and are associated with less systemic side-effects.

Quality of the evidence

Four of the studies were of high quality (Gross 2011; Rhodes 2008; Sandborn 2012; Travis 2014), with a low risk of bias across all domains. Seven studies had a higher risk of bias because of an open label design (Armuzzi 2004; Baron 1962; Bataga 2015; Raj 2014; Romano 2010; Truelove 1960a). All other publications were not deemed to be of high quality because they failed to mention if and how allocation concealment, blinding or intention-to-treat analysis occurred.

Overall the conclusions of this systematic review are limited to some extent by a substantial number of studies of poor or moderate quality. Given that the publication dates range from 1955 to 2010 this is not surprising. For the most part, earlier trials had relatively small numbers of patients and these publications often failed to indicate whether blinding, allocation concealment, and intention- to-treat analysis occurred. Furthermore it should be recognized that the concomitant standard of care varied considerably over the course of five decades resulting in an important source of clinical heterogeneity.

Potential biases in the review process

To reduce potential bias a comprehensive literature search was performed to identify all eligible studies. Additionally, Clinicaltrials.gov was searched to identify ongoing studies. Two review authors (TH, WV) independently assessed studies for inclusion, extracted data and assessed study quality. Given the relative paucity of published literature on the use of oral corticosteroids in ulcerative colitis in comparison to the many trials in CD, it is possible that studies with negative results have been performed but have never been published.

Agreements and disagreements with other studies or reviews

The findings of this systematic review are in keeping with re- cent review articles focusing on the use second-generation corticosteroids in ulcerative colitis (Danese 2014; Gionchetti 2014; Sherlock 2015) and the differences between second-generation and conventional corticosteroids (D'Haens 2016).

Similar reviews have been performed in Crohn's disease (Benchimol 2008; De Cassan 2012; Rezaie 2015; Ford 2011)

AUTHORS' CONCLUSIONS

Implications for practice

Oral corticosteroids are commonly used as induction agents in patients with moderate to severe UC. The evidence presented here suggests that oral corticosteroids are superior in inducing remission, without significantly more adverse events than placebo. Locally active corticosteroids (budesonide, beclomethasone dipropionate, fluticasone propionate and prednisolone metasulfobenzoate) are equally effective in inducing clinical remission, but appeared to be less effective in inducing endoscopic remission, although they are associated with a lower risk of adverse events as compared to systemic corticosteroids.

Implications for research

Several randomised controlled trials (Bataga 2015; D'Haens 2010; Gross 2011; Rubin 2015; Sandborn 2012; Travis 2014) have studied the efficacy and safety of budesonide in patients with UC, 5 beclomethasone dipropionate (Campieri 2003; Pica 2013; Rizzello 2002; Romano 2010; Van Assche 2015) while only two very old studies have studied the use of fluticasone (Angus 1992; Hawthorne 1993) and only one has looked into the benefits of prednisolone metasulfobenzoate (Rhodes 2008). Further research is required to assess other corticosteroids with low bio-availability such as fluticasone and prednisolone metasulfobenzoate. Future trials should re-evaluate the use of traditional corticosteroids for the induction of remission in UC since the evidence is limited to one study conducted over half a century ago (Truelove 1955). Trials a dressing corticosteroid use as an adjunctive therapy to biologicals are also desperately needed.

Oral Corticosteroids compared to Placebo for Ulcerative colitis						
Patient or population: Ulcerative colitis **Intervention:** Oral Corticosteroids **Comparison:** Placebo						
Outcomes	**Anticipated absolute effects*** **(95% CI)**		**Relative effect (95% CI)**	**№ of participants (studies)**	**Quality of the evidence (GRADE)**	**Comments**
	Risk with Placebo	**Risk with Oral Corticosteroids**				
Clinical remission	Study population		RR 2.40 (1.88 to 3.07)	1657 (7 RCTs)	⊕⊕⊖⊖ LOW	1) Considerable heterogeneity (I^2 = 59%) between study results. 2) 4/6 studies had an unclear risk of bias for allocation concealment (3 studies) or random sequence generation (1 study).
	119 per 1,000	286 per 1,000 (224 to 365)				
Clinical response	Study population		RR 1.25 (1.03 to 1.51)	1163 (5 RCTs)	⊕⊕⊕⊖ MODERATE	1) 3/5 studies had an unclear risk of bias for random sequence generation (1 study) or allocation concealment (2 studies).
	269 per 1,000	339 per 1,000 (279 to 403)				
Endoscopic remission	Study population		RR 2.01 (1.25 to 3.22)	1013 (5 RCTs)	⊕⊖⊖⊖ VERY LOW	1) Sparse number of events (N=161) 2) 4/5 had an unclear risk of bias for either allocation concealment (2 studies) or random sequence generation (2 studies) 3) Moderate heterogeneity (I^2 = 45%) between study results.
	107 per 1,000	215 per 1,000 (134 to 345)				
Endoscopic response	Study population		RR 1.19 (0.98 to 1.44)	954 (4 RCTs)	⊕⊕⊕⊖ MODERATE	1) 1/4 studies with high risk of bias (blinding)
	338 per 1,000	402 per 1,000 (331 to 486)				

Histological remission	Study population		RR 1.51 (1.12 to 2.04)	1391 (4 RCTs)	⊕⊕⊖⊖ LOW	1) Sparse number of events (N=175) 2) 2/4 studies had an unclear risk of bias for allocation concealment
	110 per 1,000	169 per 1,000 (125 to 225)				
Histological response	Study population		RR 1.71 (0.98 to 3.00)	257 (2 RCTs)	⊕⊕⊖⊖ LOW	1) Sparse number of events (N=44) 2) Low number of studies
	127 per 1,000	217 per 1,000 (125 to 381)				
Adverse events	Study population		RR 1.10 (0.99 to 1.23)	1621 (6 RCTs)	⊕⊕⊕⊖ MODERATE	1) Considerable heterogeneity in the data (I^2 = 56%)
	351 per 1,000	386 per 1,000 (348 to 432)				

***The risk in the intervention group** (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).

CI: Confidence interval; **RR:** Risk ratio

GRADE Working Group grades of evidence
High quality: We are very confident that the true effect lies close to that of the estimate of the effect
Moderate quality: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
Low quality: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
Very low quality: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

Summary of Findings Table 1 – Oral corticosteroids versus placebo for inducing remission in UC

Systemic Corticosteroids compared to Locally active corticosteroids for Ulcerative Colitis

Patient or population: Ulcerative Colitis?
Setting: Outpatients and inpatients
Intervention: Systemic Corticosteroids
Comparison: Locally active corticosteroids

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	№ of participants (studies)	Quality of the evidence (GRADE)	Comments
	Risk with Locally active corticosteroids	Risk with Systemic Corticosteroids				
Clinical Remission	Study population		RR 0.84 (0.65 to 1.08)	669 (3 RCTs)	⊕⊕⊖⊖ LOW	1) low number of observations (184 events) 2) 2/3 studies unclear risk of bias for allocation concealment. 1/3 studies unclear risk of bias for random sequence generation.
	289 per 1,000	241 per 1,000 (183 to 311)				
Clinical Response	Study population		RR 0.92 (0.78 to 1.08)	488 (2 RCTs)	⊕⊕⊖⊖ LOW	1) low number of events (N=258) 2) 2/2 studies had an unclear risk of bias for allocation concealment. 1/2 studies had an unclear risk of bias for random sequence generation
	552 per 1,000	503 per 1,000 (413 to 592)				
Endoscopic Remission	Study population		RR 0.81 (0.68, to 0.96)	680 (4 RCTs)	⊕⊕⊖⊖ LOW	1) Considerable heterogeneity (I^2 = 52%) in the data 2) 3/4 had an unclear risk of bias for allocation concealment and 1 study was also rated as unclear risk of bias for random sequence generation.
	407 per 1,000	312 per 1,000 (244 to 393)				
Endoscopic response	Study population		RR 0.98 (0.70 to 1.35)	67 (1 RCT)	⊕⊖⊖⊖ VERY LOW	1) low number of events (N=46) 2) included study had unclear risk of bias for allocation concealment 3) limited number of study (N=1)
	694 per 1,000	676 per 1,000 (429 to 855)				

Histological Remission	Study population		RR 0.50 (0.14 to 1.82)	62 (1 RCT)	⊕⊖⊖⊖ VERY LOW	1) Very low number of observation (N=9) 2) unclear risk of bias for allocation concealment 3) Limited number of studies (N=1)
	194 per 1,000	97 per 1,000 (23 to 322)				
Histological Response	Study population		RR 0.52 (0.36 to 0.74)	273 (2 RCTs)	⊕⊕⊖⊖ LOW	1) Very low number of events (N=93) 2) Both studies had unclear risk of bias for allocation concealment. 1 study had unclear risk of bias for random sequence generation
	447 per 1,000	225 per 1,000 (145 to 330)				
Adverse Events	Study population		RR 0.67 (0.54 to 0.82)	668 (3 RCTs)	⊕⊕⊖⊖ LOW	1) Sparse number of events (N=153) 2) 2/3 studies had an unclear risk of bias for allocation concealment. 1/3 studies had an unclear risk of bias for random sequence generation.
	229 per 1,000	99 per 1,000 (61 to 162)				

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CI: Confidence interval; **RR:** Risk ratio

GRADE Working Group grades of evidence
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Moderate quality: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
Low quality: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
Very low quality: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

Summary of Findings Table 2 – Systemic corticosteroids compared to locally active corticosteroids for inducing remission in UC

Oral corticosteroids compared to 5-ASA for Ulcerative Colitis						
Patient or population: Ulcervative Colitis **Setting:** Inpatients and outpatients **Intervention:** Oral corticosteroids **Comparison:** 5-ASA						
Outcomes	**Anticipated absolute effects* (95% CI)**		**Relative effect (95% CI)**	**№ of participants (studies)**	**Quality of the evidence (GRADE)**	**Comments**
	Risk with 5-ASA	**Risk with Oral corticosteroids**				
Clinical Remission	Study population		RR 0.95 (0.83 to 1.10)	1074 (7 RCTs)	⊕⊖⊖⊖ VERY LOW	1) High inconsistency in the data (I^2 = 67%) 2) 2/5 studies had a high risk of bias due to (blinding)
	425 per 1,000	402 per 1,000 (341 to 468)				
Clinical Response	Study population		RR 1.04 (0.85 to 1.27)	669 (5 RCTs)	⊕⊕⊖⊖ LOW	1) high risk of bias (blinding) in 2/5 studies 2) low number of events (N=220)
	335 per 1,000	352 per 1,000 (271 to 440)				
Endoscopic Remission	Study population		RR 0.88 (0.75 to 1.04)	427 (3 RCTs)	⊕⊖⊖⊖ VERY LOW	1) High inconsistency in the data (I^2 = 76%) 2) 2/3 studies had a high risk of bias (blinding)
	610 per 1,000	539 per 1,000 (443 to 630)				
Endoscopic response	Study population		RR 0.93 (0.82 to 1.06)	711 (2 RCTs)	⊕⊕⊖⊖ LOW	1) Very serious inconsistency in the data (I^2 = 79%)
	610 per 1,000	565 per 1,000 (480 to 643)				
Histological Remission	Study population		RR 0.67 (0.54 to 0.83)	741 (3 RCTs)	⊕⊖⊖⊖ VERY LOW	1) low number of events (N=204) 2) Serious inconsistency in the data (I^2 = 77%)
	377 per 1,000	236 per 1,000 (175 to 306)				
	Study population					1) low number of events (N=221)

Histological Response	723 per 1,000	571 per 1,000 (455 to 676)	RR 0.79 (0.67 to 0.93)	343 (1 RCT)	⊕⊕⊕⊖ MODERATE	
Adverse events	Study population		RR 1.05 (0.91 to 1.21)	959 (4 RCTs)	⊕⊕⊖⊖ LOW	1) Moderate inconsistency in the data (I^2 = 52%) 2) 1/4 studies had a high risk of bias for blinding
	340 per 1,000	362 per 1,000 (294 to 438)				

*__The risk in the intervention group__ (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).

CI: Confidence interval; **RR:** Risk ratio

GRADE Working Group grades of evidence
High quality: We are very confident that the true effect lies close to that of the estimate of the effect
Moderate quality: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
Low quality: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
Very low quality: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

Summary of findings table 3 – Oral corticosteroids compared to 5-ASA for inducing remission in Ulcerative Colitis

High dose corticosteroids compared to low dose corticosteroids for inducing remission in Ulcerative Colitis						
Patient or population: Patients with UC **Intervention:** High dose corticosteroids **Comparison:** low dose corticosteroids						
Outcomes	**Anticipated absolute effects[*] (95% CI)**		**Relative effect (95% CI)**	**№ of participants (studies)**	**Certainty of the evidence (GRADE)**	**Comments**
	Risk with low dose corticosteroids	**Risk with High dose corticosteroids**				
Clinical Remission	Study population		RR 1.56 (1.17 to 2.08)	801 (5 RCTs)	⊕⊖⊖⊖ VERY LOW	1) High inconsistency in the data (I2 = 87%) 2) Low number of events (N=193) 3) 2/5 studies had high risk of bias in regard to blinding
	180 per 1,000	282 per 1,000 (211 to 375)				
Clinical Response	Study population		RR 1.27 (1.01 to 1.60)	680 (4 RCTs)	⊕⊕⊖⊖ LOW	1) Low number of events (N=222) 2) 2/4 studies had high risk of bias in regard to blinding
	287 per 1,000	364 per 1,000 (290 to 459)				
Endoscopic Remission	Study population		RR 0.91 (0.64 to 1.30)	77 (1 RCT)	⊕⊕⊖⊖ LOW	1) Low number of events (N=48) 2) Evidence based on 1 study only
	650 per 1,000	592 per 1,000 (416 to 845)				
Endoscopic response	Study population		RR 1.36 (1.07 to 1.73)	576 (2 RCTs)	⊕⊕⊕⊖ MODERATE	1) Low number of events (N=206)
	300 per 1,000	407 per 1,000 (321 to 518)				
Histological remission	Study population		RR 1.04 (0.37 to 2.96)	576 (2 RCTs)	⊕⊕⊖⊖ LOW	1) Low number of events (N=58) 2) Significant inconsistency in the results (I^2 = 64%)
	80 per 1,000	83 per 1,000 (30 to 237)				
Adverse Events	Study population					

	601 per 1,000	577 per 1,000 (517 to 649)	RR 0.96 (0.86 to 1.08)	801 (5 RCTs)	⊕⊕⊕⊖ MODERATE	1) 2/5 studies had high risk of bias in regard to blinding

*__The risk in the intervention group__ (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).

CI: Confidence interval; **RR:** Risk ratio; **OR:** Odds ratio;

GRADE Working Group grades of evidence
High certainty: We are very confident that the true effect lies close to that of the estimate of the effect
Moderate certainty: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
Low certainty: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
Very low certainty: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

Summary of findings table 4 – Comparing high dose corticosteroid regiments to low dose for inducing remission in ulcerative colitis

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* *Indicates the major publication for the study*

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

Fibrostenosis is a frequent complication of longstanding CD affecting up to one third of patients.[1] It is characterised by the accumulation of fibrotic tissue in the bowel wall eventually leading to intestinal lumen narrowing and mechanical obstruction. Despite recent advances in anti-inflammatory therapy, the only therapeutic option to treat intestinal fibrosis remains surgical removal of the affected bowel segment. Unfortunately, up tot 70% of patients will experience recurrent fibrostenosis after surgery and its associated loss of viable intestinal tissue renders surgery an undesired option in the long term management of these patients.[2] Currently, there are no anti-fibrotic agents available partly due to a lack of understanding of the pathophysiology of intestinal fibrosis, but also because of difficulties in constructing clinical trials.[2-4] Indeed, both identifying patients at risk to include in these trials as well as finding good disease markers for therapeutic follow-up has been proven difficult. Inspired by these difficulties the main goals of this project were dual: first of all to evaluate a possible new anti-fibrotic strategy in CD-related fibrosis, by inhibiting Rho kinases, and secondly to identify new disease markers usuable in clinical trials addressing intestinal fibrosis.

1. Treatment of intestinal fibrosis in experimental inflammatory bowel disease by the pleiotropic actions of a local Rho kinase inhibitor

In the first part of the results section of this thesis (**Chapter III.1**) evidence was given for the use of local Rho kinase inhibitors in the treatment of CD-related intestinal fibrosis.

Rho kinases are small serine/threonine kinases involved in cytoskeleton organisation and play an important role in several processes involved in fibrogenesis including fibroblast activation and migration, EMT, autophagy, and mechanosensing of the matrix stiffness. Therefore they represent attractive targets for anti-fibrotic therapy and have been succesfully used in other organ systems, including experimental models of pulmonary fibrosis, renal fibrosis, hepatic and cardiac fibrosis.[5 6-10]

In this chapter, we showed that Rho kinases are involved in CD-related fibrosis as well. Enzyme activity is enhanced in ileal biopsies taken from CD patients with active ileitis and in stenotic segments compared to the ileum of normal, healthy subjects. Interestingly, biopsies taken from non-stenotic segments within the same CD patient did not show an increased Rho kinase activity. These findings not only suggest a role of Rho kinase activity in intestinal fibrosis but also provide us with a possible time frame when these kinases might play their role during fibrogenesis. Rho kinase activity starts and is at its highest during active inflammation. However, for reasons not completely understood Rho kinase activity remains high in the segments that develop fibrostenosis even though inflammation has

subsided. One explanation might be an increased matrix stiffness due to repeated inflammation in these segments, which has been shown to activate the Rho kinase system.[11,12] Other authors have shown similarly increased Rho kinase activity in the inflamed ileum of CD patients, however here we provided the first evidence that Rho kinases are also involved in CD-related intestinal fibrosis.[13] Interestingly, many cells involved in fibrogenesis express this Rho kinase activity, including epithelial cells, endothelial cells, fibroblasts (both subepithelial and located within the submucosa) and smooth muscle cells.

Given the involvement of Rho kinases in intestinal fibrosis and the efficacy of their inhibitors in other organ systems, the rationale for Rho kinase inhibitors in CD-related fibrosis is substantial. However, cardiovascular side-effects such as systemic hypotension limit their clinical applicability. To circumvent this, a collaboration with Amakem (Diepenbeek, Belgium) was started to develop a locally active Rho kinase inhibitor that specifically targets the gut. Several strategies have previously been described for localizing drug exposure to the intestine, including low intestinal permeability due to physical-chemical properties, the targeting of efflux transporters, by increasing a drugs lipophilicity to promote high systemic clearance, or by using soft drugs (which are degraded rapidly into inactive metabolites).[14] Eventually, AMA0825 was selected, a potent (e.g. 100x higher affinity then Y27632) soft pan-ROCK inhibitor with mimimized systemic exposure due to 1) rapid hydrolysis by paraoxonases upon contact with the portal circulation and 2) a high first pass effect in the liver. In a set of pharmacokinetic experiments, oral administration of AMA0825 resulted in adequate drug concentrations in the colon up to 24 hours after administration while plasma concentrations were not in the detectable range at any timepoint. In line with these results, administration of AMA0825 in spontaneous hypertensive rats did not result in a drop in arterial blood pressure as systemic Rho kinase inhibitors have previously been shown to induce in these animals.[15] However, these data were acquired in healthy animals and absorption of drugs can be altered when intestinal inflammation is present. In the adoptive T cell transfer model, a commonly used model of CD-like inflammation, administration of AMA0825 lowered Rho kinase activity in the colon but did not affect other organ systems confirming a localized action even in a diseased state.

In the same chapter, local Rho kinase inhibition by AMA0825 was shown to effectively prevent intestinal fibrosis in several murine models, both in monotherapy and in combination with anti-TNF. Interestingly, this preventive effect was independent of an anti-inflammatory effect (suggested by unaltered degree of inflammation in the chronic DSS model and consequently confirmed in acute models of inflammation including acute DSS and TNBS), suggesting direct anti-fibrotic effects of

AMA0825. This is in contradiction with earlier studies reporting on the anti-inflammatory effects of Rho kinase inhibitors in murine models of inflammatory bowel disease.[13] However, these studies were performed using a non-selective Rho kinase inhibitor Y27632, which has been shown to inhibit other kinases involved in inflammation including PKC-related kinase-2, protein kinase N and citron kinase in similar concentrations.[16,17] Moreover, in a set of *in vitro* experiments we showed that Y27632 downregulated inflammatory cytokine production from epithelial and endothelial cells in concentrations which did not affect Rho kinase activity in these cells suggesting off-target effects.

Although there was no evidence of an anti-inflammatory effect of AMA0825, the observation that treated mice in both the acute DSS and TNBS model had a better weight evolution, was discordant. One possible explanation might lie in the fact that Rho kinase is involved in leptin metabolism, a hormone involved in hunger regulation, and Rho kinase deficient mice are known to have increased body weight compared to their wildtype siblings. Future studies should measure food intake of treated mice and measure circulating leptin levels to further explore this notion.[18]

Given the absence of anti-inflammatory effects, combining AMA0825 with anti-inflammatory agents makes clinical sense. Combination therapy with anti-TNF in the adoptive T cell transfer model resulted not only in suppression of inflammation but additionaly prevented accumulation of fibrotic tissue in comparision to anti-TNF therapy alone. Another benefit of combining TNF antagonists with AMA0825 might lie in the suppression of MMP-3 and -12 (shown in a set of *ex vivo* experiments using biopsies of CD patients with fibrostenotic disease) as proteolytic degradation of TNF antagonists by MMP-3 and -12 has been causally linked to anti-TNF refractory disease.[19] Combination therapy might thus not only suppress inflammation and prevent fibrostenotic complication but might also protect against TNF antagonist failure, although further studies are needed to address this issue.

As approximately 10% of patients will already have fibrostenosing disease at the moment of diagnosis, the ideal anti-fibrotic agent does not only prevent but also regresses fibrosis.[2] In a chronic DSS model with variable treatment starting times, AMA0825 reversed already established fibrosis. Additionally, in an *in vitro* set of experiments AMA0825 added to cell which were stimulated for 48 hours with TGFβ1 significantly reduced IL6 production, further underlining its ability to reverse profibrotic effects. Combination therapy may not alone stabilise disease in these patients but also help avoid surgery.

Other preclinical anti-fibrotic agents have been studied in experimental IBD (for an overview see Table 2 – Introduction – p 72). However, most of these agents interfere with TGFβ/SMAD signaling which,

given the anti-inflammatory properties of TGFβ/SMAD might not be the best option.[20] Indeed, a phase III clinical trial with SMAD7 antisense oligonucleotide (Mongersen), was ended prematurely because of lack of efficacy despite very promising remission rates in the phase II trial.[21] The local Rho kinase inhibitor AMA0825, however, interferes with TGFβ-induced (myo)fibroblast and smooth muscle cell activation in a SMAD-independent manner (Figure XX).

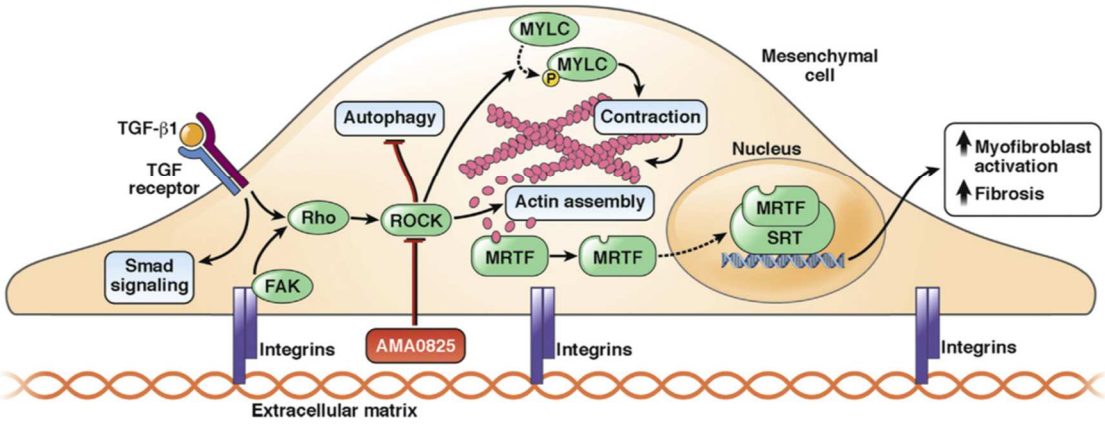

Figure 1 – Working model for AMA0825-induced ROCK inhibition in intestinal fibrosis. FAK = focal adhesion kinase, MYLC = myosin light chain, MRTF = myocardin-related transcription factor, ROCK = rho associated kinase, SMAD = mothers against decapentaplegic, SRT = serum response transcription factor, TGF = transforming growth factor. [Adapted from Rieder et al.[22]]

Mechanistically, AMA0825 inhibits the Rho/ROCK system of mesenchym cells including fibroblasts and smooth muscle cells. Rho/ROCK is activated through direct phosphorylation by the TGF receptor in a SMAD-independent manner (See Figure 1). Additionally, focal adhesion kinases (FAK), which sense the extracellular matrix stiffness through integrin-mediated contacts, can also activate the fibroblast Rho/ROCK pathway. Rho kinase inhibition interferes with actin assembly, essential for stress fiber formation and keeps MRTF sequestered to the cytoplasm, preventing transcription of various profibrotic factors under the control of the serum response transcription factor (SRT) including *MMP2*, *ACTA2* and *TGFβ1*. Additionally, AMA0825 interferes with myosin light chain (MYLC) phosphorylation, involved in cytoskeleton contraction and mesenchymal cell motility. Although we are the first to report on the use of Rho kinase inhibitors in intestinal fibrosis, in line with our results other authors have reported on the succesful inhibition of colonic fibroblast activation using selective MRTF inhibitors.[12] However, Rho kinase inhibitors act more broadly and influence processes not controlled by MRTF but essential to fibrogenesis as explained below.

Aside from interfering with (myo)fibroblast/smooth muscle cell activation, AMA0825 was for instance also shown to interfere with EMT, a transformational process in which epithelial cells transition into fibroblasts, thereby reducing fibroblast influx. This process could additionally explain the reduced number of myofibroblasts seen in our chronic DSS model. In addition, we were able to show an increased number of apoptotic myofibroblasts in the colon of mice receiving AMA0825. However, as we were unable to induce fibroblast apoptosis *in vitro* the exact cellular mechanisms of increased myofibroblast apoptosis following AMA0825 administration remain unknown. As this could be one of the vital mechanisms involved in reversal of fibrosis, further studies are warranted, especially as in pulmonary fibroblasts, Rho kinase inhibition-induced apoptosis has been shown to play an essential role in fibrosis reversal.[8]

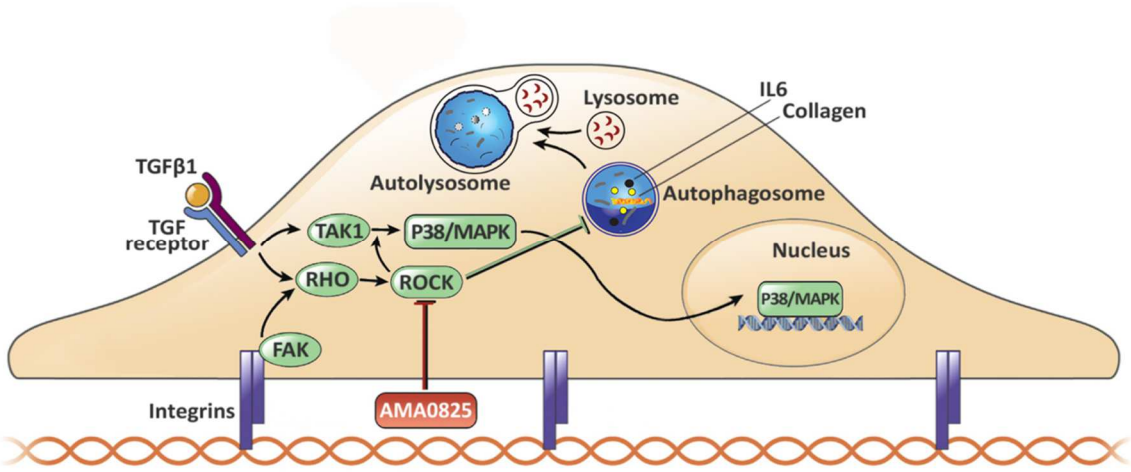

Figure 2 – AMA0825 increases autophagy-mediated break-down of IL6 and collagen contributing to its anti-fibrotic effect. FAK = focal adhesion kinase, p38/MAPK = mitogen activated protein kinase, ROCK = rho associated kinase, TAK1 = Transforming growth factor beta associated kinase, TGF = transforming growth factor.

Increasing autophagy in intestinal fibroblasts is another mechanism by which AMA0825 impairs fibrogenesis and is involved in the effects of Rho kinase inhibition on fibroblast IL6 and collagen production (See Figure 2). As adding a pharmacological inhibitor of autophagy (Bafilomycin A1) interfered with AMA0825 effects on both IL6 transcription and secretion, an upstream mediator which is targeted by the AMA0825-induced increased autophagy response is suspected. p38/MAPK is the most likely candidate as it controls IL6 release in intestinal fibroblasts and autophagy inhibition increases phopho-p38 levels. Alternatively, p62, which is degraded in autophagosomes, could be involved as it is a known activator of p38/MAPK signalling.[23] Conversely, only AMA0825-induced alterations to collagen secretion and not mRNA transcription were hampered by autophagy inhibition, suggesting increased posttranslational degradation of collagen in the autophagasomes as one of the

AMA0825 mechanisms of action. In a set of immunofluorescence experiments we were able to show an increased presence of collagen in the autophagasomes of AMA0825-treated cells supporting this hypothesis. The importance of the increased autophagic response was nicely illustrated in intestinal fibroblasts isolated from *Atg16l1*$^{hm/hm}$ mice that are unresponsive to the effects of Rho kinase inhibition on TGFβ-induced IL6 secretion. Beside clarifying the mode of action of Rho kinase inhibition, these set of experiments also provided better insights into the role of autophagy in fibrogenesis. Although controversial, it is generally believed that fibrogenesis can be halted by inducing autophagy. This study has provided additional evidence to this paradigm. Future studies should further explore the role of autophagy in intestinal fibrosis.

AMA0825 is a pan-Rho kinase inhibitor inhibiting both Rho kinase 1 (ROCK1) and ROCK2. However, selective ROCK1 and ROCK2 inhibitor are becoming increasingly available and might be associated with less side-effects upon systemic exposure.[24] Currently it is unclear wether ROCK1 or ROCK2 inhibition alone is sufficient or if both need to be supressed for maximal efficacy. Theoretically, ROCK2 seems to be the safest option as it is only expressed in smooth muscle and neuronal cells.[25] However, ROCK1 appears to be most involved in fibrogenesis with ROCK1 deficient mice developing less pronounced renal fibrosis in a murine UOO model while ROCK2 deficiency offers no protection. [26,27] In cardiac fibrosis, however both specific targeting of ROCK1 and 2 ameloriated cardiac fibrosis in several models. [28-33] Future studies should focus on the specific contributions of ROCK1 and 2 in intestinal fibrosis.

Besides preventing intestinal fibrosis, Rho kinase inhibition might also be beneficial in IBD-related colorectal cancer. In a orthotopic murine model of colorectal carcinoma, Rho kinase inhibition reduced metastasis of colorectal xenograft tumors by SMAD-independent inhibition of BMP signalling. Additionally, Rho kinases play an important role in cancerogenesis and metastasis in other organ systems making them attractive targets for anti-cancer therapies.[34] Future studies should investigate the effects of Rho kinase inhibition in murine models of IBD-related cancer, e.g. by using the azoxymethane-DSS model.[35]

Despite the promising profile of AMA0825 in preventing and treating intestinal fibrosis, constructing a clinical trial to translate our findings to the human setting might prove difficult. Several issues arise when designing such a trial. First of all, identification of the most suitable population is problematic. As only 30% of CD patients will develop fibrostenosis over time, a huge number of patients is required to achieve enough events. Moreover hard endpoints like surgery need a long time of observation.

Therefore predictors for fibrostenotic CD phenotype and biomarkers of intestinal fibrosis would be useful in the construction of clinical trials but are currently not available . The second part of this work was focused on this topic.

2. Multi-locus genetic risk for early development of fibrostenosis in patients with Crohn's disease

In the second part of the results section of this thesis (**Chapter III.2**) genetic risk factors for early fibrostenotic CD were investigated. This genetic association study, using a well-phenotyped population based on suggestive findings of fibrostenosis on cross-sectional imaging (CT/MRI enterography) provided evidence for a genetic contribution to early fibrostenotic CD.

Some of the identified SNPs in this study also pointed towards new disease pathways that are possibly involved in fibrostenosing CD. The most significantly associated SNP (rs35223850) for instance was located within the *MIS18BP1* gene encoding a component of the centromere and essential for proper chromosome segregation during mitosis. [36,37] Although this is the first study to link centromere abnormalities to intestinal fibrosis, other studies have found transcriptional upregulation of the gene in mouse models of renal fibrosis.[38] Together with our finding that the gene is upregulated in fibroblasts isolated from stenotic CD bowel segments, *MIS18BP1* is an interesting candidate gene for further functional studies. Other interesting pathways that were linked to early fibrostenosis in this study were the epidermal growth factor receptor (EGFR - rs4947982) involved in mechanosensing of extracellular matrix resistance and cadherin-4 (CDH4 - rs4925207), belonging to a family of proteins involved in EMT. CDH4 was also found to be upregulated in models of cardiac fibrosis.[39-41] Future studies should explore the functional relevance of these genes in intestinal fibrosis e.g. by creating fibroblast-specific knock-out mice and subjecting them to chronic DSS. This can be achieved through the Cre-Lox recombination system using the fibroblasts-specific promotors collagen 1A2 (Col1a2) or growth differentiation factor (GDF) 5. The link with Glutathion peroxidase-4 (GPX-4) is also potentially a very interesting one, for the first time linking the process of ferroptosis to intestinal fibrosis. Ferroptosis is a form of regulated cell death resulting from accumulation of lipid peroxidation products and reactive oxygen species (ROS) derived from iron metabolism.[42] As GPX4 functions as an important negative regulator of ferroptosis (by reducing lipid hydroperoxides and ROS production), overexpression of GPX4 in intestinal fibroblasts could prevent ferroptic cell death in these cells and therefore promote fibrogenesis. This hypothesis could be tested by performing a chronic DSS

experiment in fibroblast-specific GPX4-KO mice or by administrating lipid peroxidation inhibitors such as ferrostatin to mice undergoing chronic DSS.

Other SNPs (rs9325636, rs12072417, rs9960012, rs7406291, rs1485470, rs6040339) were not located within known genes and their relation to possible disease pathways is less obvious. One possibility is that the identified SNPs are in linkage disequilibrium (LD) with another causal polymorphism. However, no other SNPs were found to be in LD with the polymorphisms identified in this study. The found SNPs can also influence other genes by for example epigenetic modifications or be a proxy for causative genes located at a distance. According to a recent study exploring the distance between SNPs and causative genes in GWAS found that the affected genes can be located up to 2 Mbp away from the identified SNP.[43] Table 1 gives an example of several genes that have a possible pathophysiological link with intestinal fibrosis and are located within close proximity of SNPs identified in this study. High-throughput sequencing of the genomic regions surrounding these SNPs could help determine whether these genes are actually involved in the genetic risk for early fibrostenosis.

SNP ID	Chr	Gene-snp distance (bp)	Gene Symbol	Encoded protein	Function	Possible link with intestinal fibrosis
rs17554931	19	140	*GPX4*	Glutathion peroxidase 4	Protection against oxidative stress, inhibits ferroptosis	Hypermethylated in murine liver fibrosis [39]
		8,394	*POLR2E*	RNA polymerase II subunit E	mRNA transcription	Upregulated in murine renal fibrosis[38]
		3,848	*SBNO2*	Strawberry Notch 2 homologue	Involved in downstream IL10 signaling	Identified as CD susceptibility gene[44]
		15,955	*HMHA1*	Minor histocompatibility protein HA-1	MHC stability	Act as a Rho GTPase activating protein[45]
		64,718	*CNN2*	Calponin 2	Actin stabilization	Involved in fibroblast migration, expression regulated by mechanical tension and surface stiffness[46]
rs4925207	20	0	*CDH4*	Cadherin 4	Cell-cell adhesion	Upregulated in hypoxia-induced cardiac fibrosis in rats[41]
		82,582	*TAF4*	Transcription initiation factor TFIID subunit 4	mRNA transcription initiation	Involved in retinoic acid-induced fibroblast activation[47] TAF4 inactivation activates TGFβ signaling in fibroblasts[48]
rs35223850	14	0	*MIS18BP1*	MIS18 binding protein 1	Centromere maintenance	Upregulated in murine renal fibrosis[38]
		23,555	*FANCM*	Fanconi anemia, comp-lementation group M	DNA repair	Unknown
		161,626	*PERP*	p53 apoptosis effector related to PMP-22	Involved in p53 induced apoptosis	Unknown
rs12072417	1	5,790	*OLFML2B*	Olfactomedin-like 2b	ECM protein	Involved in EMT in chick embryos[49]

		39,536	*NOS1AP*	Nitric oxide synthase 1 adaptor protein	Involved in NO signaling	Unknown

Table 1 – Overview of genes in close proximity to SNPs associated with early fibrostenotic CD

Conversely, our study provided evidence against some of the earlier genetic associations made by less-well phenotyped studies. While a German population-based study found a relationship between polymorphisms in the *IL23R* gene and fibrostenotic disease, our study could not replicate these results.[50] Although early fibrostenotic disease was initially associated with rs116630177 in our cohort, the association was lost when correcting for disease location suggesting that variants in the *IL23R* gene more likely predispose for ileal disease. Similarly, in this selected population of CD patients with ileal or ileocaecal disease (Montreal L1 or L3) NOD2 variants were distributed equally between cases and controls, again suggesting an association with disease location more than fibrostenosis in itselfs. These findings are in line with those from the largest, international genotype-phenotype published in IBD.[51]

Based on the identified SNPs in this study, a genetic risk score (GRS) was calculated which was able to discriminate accurately between CD patients with fibrostenotic complications and those with a benign disease course without these complications (AUC 0.885 in the discovery cohort). Using a cut-off value based on the discovery cohort yielding 80% sensitivity and 81% specificity to predict fibrostenosis in the validation cohort, resulted in an OR of 2.65 for fibrostenotic complications with a sensitivity of 51% and a specificity of 72%. Although this is not accurate enough to base clinical management on this risk score alone, combination with future (bio)markers could perhaps be clinically useful. At this moment, this genetic risk score holds potential as as a stratification tool for inclusion of patients in clinical trials.

In this retrospective study, there was no relation between GRS and the velocity with which the fibrostenotic complications developed. However, patients with a high GRS developed fibrostenosis considerably faster compared to patients with a low GRS, suggesting an important pathophysiological role for the SNPs identified in this study.

On of the major strengths of this study was the fact that the results generated in this study were replicated in an independent cohort of fibrostenotic CD patients. Assocations seen were in line with the ones seen in the original cohort, strengthening our findings. Replication in larger cohorts, however, should provide even stronger evidence for these associations.

Usage of the Illumina Immunochip forms a possible limitation to this study. As the Immunochip is specifically designed for detecting associations with-immune-related genes, important associations with inflammation-unrelated fibrogenesis pathways might have been missed. It could be interesting to repeat the study design using GWAS data.

In conclusion, in this part of this work we identified several genetic risk factors for early fibrostenotic CD. However, the risk increase associated with the individual SNPs is too small and their population prevalance too low to be useful by themselves as a predictive factor. Combining presence of the variants identified in this study does give useful information about evolution to fibrosis over time, but should be replicated in larger cohorts.

3. Biomarkers for intestinal fibrosis

In the last chapter of this thesis (**Chapter III.3**) we explored possible biomarkers for intestinal fibrosis. Finding a serum biomarker could be a great asset for constructing clinical trials investigating the efficacy of anti-fibrotic therapies in CD. Additionally, fibrosisbiomarkers could help differentiate between inflammatory strictures (that can be treated by upscaling medical therapy) or fibrotic strictures (that should be referred to the surgeon). Ideally, a biomarker would allow for prediction of future fibrostenotic complications before they arise.

In this preliminary study, biomarkers were studied in a well-phenotyped population of fibrostenotic CD, based on CT and/or MRI imaging. Serum levels of MMP-2, MMP-3 and TIMP-3 were found to be differentially expressed between patients with fibrostenotic and uncomplicated CD. MMP-2 and -3 belong to the matrix metalloproteinases, a family of zinc and calcium-dependent endopeptidases involved in extracellular matrix degradation and were lower in patients with fibrostenotic complications. Conversely, serum levels of TIMP-3, an inhibitor of MMPs, was higher in these patients. In this cohort, each one of these serum markers alone had a decent discriminant function for predicting fibrostenosis, at least in patients with evidence of inflammation (defined as CRP ≥ 5 and/or presence of intestinal inflammation on endoscopy/radiology). Interestingly however, a ratio of these three markers (MMP-2 * MMP-3/TIMP-3) outperformed the individual markers with an AUC of 0.855. A cut-off value lower than 2328 was in this population associated with a positive and negative predictive value of respectively 82.4% and 86.7%. These results are comparable to MRI-based techniques using delayed gadolinium-enhancement as a surrogate marker for fibrostenosis.[52] However, a simple biomarker (or combination of biomarkers) holds obvious advantages over

expensive, time-consuming and not readily available techniques such as gadolinium-enhanced MRI and does not require a specially-trained radiologist.

No other studies have investigated circulating levels of these MMPs and TIMPs in fibrostenotic CD. One study found differential expression of TIMP-1 in the mucosa overlying fibrostenotic strictures but did not observe any differences in serum levels, a finding that is confirmed in the present study.

It is unclear as to why these markers perform better in the presence of inflammation. However, it is in these settings when they would be most useful. Determining if a stenosis is inflammatory or fibrotic is most difficult in patients with active disease, especially on CT/MRI imaging. Determining these additional markers of fibrostenosis in this setting might help clinical decisions.

In this study, samples predating the development of fibrostenotic complications were available (N=16). Interestingly, MMP-10 levels were able to discriminate between patients who would develop fibrostenosis later in life compared to patients who experienced an uncomplicated disease course with serum levels tending to be lower in patients who were at risk for developing fibrostenosis.

Biomarkers that can predict fibrostenosis before it occurs are even more valuable as they allow for preventive measures. In our preliminary study, 16 patients with serum samples collected before fibrostenotic complications occurred were included. Only MMP-10 levels could discriminate these patients from CD patients with a more benign, inflammatory disease course, with serum levels tending to be lower in patients who developed fibrostenosis (AUC of 0.716). If confirmed in prospective studies, this could signal a group of patients who would be eligible for intensified therapy to prevent these complications from developing.

N-glycolysation profiles have shown promising results in predicting liver fibrosis. [53-55] In this study focussing on fibrostenotic complications of CD, this did not appear to be the case. As our panel used was specifically developed for identifying liver fibrosis, repeating the study with an unrestricted glycomics panel could be useful. An exploratory study in 28 patients comparing serum samples before and after surgery found differential expression of hepatic growth factor a and cartilage oligometrix matrix protein, illustrating that serum glycomics might still be an interesting contender in the search for a fibrosis biomarker in CD.[56] Other hypothesis-free strategies such as serum proteomics or metabolomics could also provide interesting new markers.

Being an exploratory study, our results should be interpreted with caution and need to be confirmed in larger validation sets and prospective studies. Further studies could additionally look at changing serum profiles following intestinal surgery to identify potential new markers.

4. Oral corticosteroids for induction of remission in Ulcerative Colitis

In the last chapter of this thesis (Chapter III.4) another controversial item in the management of IBD was assessed. Current guidelines situate oral corticosteroids (both systemic and locally active ones) as a second-line therapy following failure of 5-ASA treatment and they are widely used for the treatment of mild- to moderate UC. [57] However, these recommendations are mainly based on randomized clinical trials dating back to the 1950s. In this chapter, a systematic review and meta-analysis was performed listing all the available evidence for the use of corticosteroids in the induction of remission in UC following the Cochrane methodology.

First, in this chapter, by combining the evidence from 7 randomized clinical trials oral corticosteroids were shown to achieve better clinical, endoscopic and histological remission rates compared to placebo. Although in line with clinical experience, this finding is important from an evidence-based medicine point of view. The fact that oral corticosteroids were also able to induce histological remission is new and goes against the general preconception that corticosteroids only provide symptomatic relief. Interestingly, no increased risk of adverse events was found, although the number of adverse events was numerically higher in the corticosteroid treated group. The fact that most of the included studies (6/7) were performed using locally active corticosteroids (which are presumed and proven further in this study to have lesser adverse events than traditional systemic corticosteroids). Also, the only study that investigated systemic corticosteroids dates back from the 1950s raising concerns about the consistency of reporting adverse events in these older trials.

In the second part of this study, the effects of systemic corticosteroids (e.g. methylprednisolone) and locally active corticosteroids (e.g. budesonide) were compared. Both groups appeared to be equally as effective in inducing clinical remission, although systemic corticosteroids seem to hold a slight advantage in inducing endoscopic remission. Adverse events, however, were fewer with the locally active formulations. By combining the evidence from 4 studies, this meta-analysis confirms current guidelines in recommending the use of locally active corticosteroids over systemic formulations but reserving the latter ones for more serious disease flares.

Thirdly, oral corticosteroids were compared to other active treatments for UC. In all of the UC guidelines, 5-ASA is regarded as first line therapy.[57] Combining data from eight studies, oral corticosteroids were similar in inducing clinical and endoscopic remission to 5-ASA, with 5-ASA inducing histological remission more frequently. This is somewhat surprising considering the fact that corticosteroids are positioned as second-line therapy in patients failing 5-ASA treatment. Of note, however, that none of these studies were set up to evaluate the performance of corticosteroids in the event of 5-ASA failure. Secondly, most of the studies included in this part of the analysis had low GRADE ratings and results should be interpreted with caution. Lastly, presumably these results mainly reflect a milder disease spectrum, in which it is plausible to assume that corticosteroids hold no advantage over 5-ASA. An interesting finding from this study is the fact that 5-ASA appears to be more effective in inducing histological remission, a finding that should be investigated more thoroughly by prospective studies.

A limitation of the applicability of the current meta-analysis is the large number of studies of low quality that were included. As many of the included studies (especially regarding the use of systemic corticosteroids) date are considerably older, this is not surprising as standards of study outcome reporting have become more rigorous with time.

In conclusion, this last part of this work provides some clarity in another difficult issue in the management of IBD patients and advocates the use of oral corticosteroids (both systemic and locally active) for the induction of remission in UC.

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CHAPTER V

ADDITIONAL PUBLICATIONS

CURRICULUM VITAE

Curriculum Vitae

PERSONALIA

Name	*Tom Holvoet*
Adress	*Teerlingstraat 32 2B* *9880 Aalter*
Cell Phone	*0474/92.69.04*
Email	*T.Holvoet@UGent.be*
Nationality	*Belgian*
Date of Birth	*24/07/1986*

EDUCATION

1998 – 2004	*Latijn Wiskunde* *Heilig Hartcollege Waregem*
2004 – 2007	*Bachelor of Science in Medicine* *Ghent University* *Summa Cum Laude*
2007 – 2011	*Master of Science in Medicine* *Ghent University* *Summa Cum Laude*
	Winner price Pharmacien et Doctororesse Nedeljkovitch 2011
	Winner price Vereniging der Geneesheren Oud-Studenten Universiteit Gent
2008	*Postgraduate course "Principles of Electrocardiography"*
2011 – 2017	*Master na master in Specialistic Medicine: Internal Medicine*
	Ghent University
2013 – 2017	*PhD fellowship (Research Foundation Flanders)*
	Promotor: Prof. Dr. De Vos, *Ghent University*
2017 – present	*Master na master in Specialisatic Medicine: Gastroenterology*
	Ghent University

CLINICAL EXPERIENCE

October 2011 – September 2012	Resident– Internal Medicine *AZ Groeninge, Kortrijk, Belgium*
October 2012 – September 2013	Resident – Internal Medicine *Heilig Hart Ziekenhuis, Roeselare, Belgium*
October 2013 - September 2017	PhD fellowship (Research Foundation Flanders) *Ghent University, Ghent, Belgium*
October 2017 – Present	Resident – Gastroenterology *AZ Sint Nikolaas, Sint Niklaas, Belgium*

SCIENTIFIC ACTIVITIES

MASTERTHESIS

2007 – 2009	*Functionele parameters voor en na radicale therapie bij thoracale tumoren* *Prof. Dr. Derom, Prof. Dr. Van Meerbeeck*
2010- 2011	*Genetic risk factors and clinical implications of Staphylococcus aureus carriership* Promotors: Prof. Dr. Brusselle (UGent); Prof. Dr. Strycker (Erasmus Rotterdam)

ARTICLES

A1 PAPERS

1. Quantitative Perfusion Scintigraphy or Anatomic Segment Method in lung cancer resection.
   Holvoet T, Van Meerbeeck J, Van De Wiele C, Salhi B, Derom E.
   Lung Cancer 2011;74:212-8 ***(IF 4.294)***

2. Systematic Review of Guidelines for Management of Intermediate Hepatocellular Carcinoma Using the Appraisal of Guidelines Research and Evaluation II Instrument
   Holvoet T, Raevens S, Vandewynckel Y, Van Biesen W, Geboes K, Van Vlierberghe H.
   Dig Liver Dis 2015;47:877-83 ***(IF 3.061)***

3. Assesment of faecal microbial transfer in irritable bowel syndrome with severe bloating
   Holvoet T, Joossens M, Wang J, Boelens J, Verhasselt B, Laukens D, Van Vlierberghe H, Hindryckx P, De Vos M, De Looze D, Raes J.
   Gut 2017; 6(5):980-982 ***(IF 16.658)***

4. Treatment of intestinal fibrosis in experimental inflammatory bowel disease by the pleiotropic actions of a local Rho kinase inhibitor
   Holvoet T, Devriese S, Castermans K, Boland S, Leysen D, Vandewynckel Y, Devisscher L, Vandenbossche L, Van Welden S, Dullaers M, De Rycke R, Geboes K, Bourin A, Defert O, Hindryckx P, De Vos M and Laukens D.
   Gastroenterology 2017;153:1054-67 ***(IF 18.392)***

5. Haematopoetic prolyl hydroxylase-1 deficiency promotes M2 macrophage polarization and is both necessary and sufficient to protect against experimental colitis
   Van Welden S, De Vos M, Wielockx B, Tavernier S, Dullaers M, Neyt S, Deschamps B, Devisscher L, Devriese S , Vandenbossche L, **Holvoet T**, Baeyens A, De Vos F, Correale C, D'Alessio S, Vanhove C, Verhasselt B, Elewaut D, Breier G, Janssens S, Carmeliet P, Danese S, Laukens D , Hindryckx P
   J Pathol 2017; 241:547-58 ***(IF 6.894)***

6. T84 monolayers are superior to Caco-2 as a model system for colonocytes
   Devriese S, Vandenbossche L, Van Welden S, **Holvoet T**, Hindryckx P, De Vos M, Laukens D
   Histochem Cell Biol 2017;148:85-93 ***(IF 2.553)***

7. Disease Activity Indices in Coeliac Disease: Systematic Review and Recommendations for Clinical Trials
   Hindryckx P, Levesque B, **Holvoet T**, Durand S, Tang CM, Parker C, Khanna R, Shackelton L, D'Haens G, Sandborn W, Feagan B, Lebwohl B, Leffler D, Jairath V
   Gut 2018;67:61-69 ***(IF 16.658)***

8. Ursodeoxycholic Acid and Its Taurine- or Glycine-Conjugated Species Reduce Colitogenic Dysbiosis
   and Equally Suppress Experimental Colitis in Mice.
   Van den Bossche L, Hindryckx P, Devisscher L, Devriese S, Van Welden S, **Holvoet T**, Vilchez-Vargas R, Vital M, Pieper DH, Vanden Bussche J, Vanhaecke L, Van de Wiele T, De Vos M, Laukens D
   Appl Environ Microbiol 2017;87: e02766-16 ***(IF 3.668)***

9. Tauroursodeoxycholic acid protects bile acid homeostasis under inflammatory conditions and damp
   ens Crohn's disease-like ileitis.
   Van den Bossche L, Borsboom D, Devriese S, Van Welden S, **Holvoet T**, Devisscher L, Hindryckx P, De Vos M, Laukens D.

   Lab Invest 2017;97: 519-29 ***(IF 4.857)***

NON A1 PAPERS

1. Aanpak van diverticulitis: nieuwe richtlijnen.
   Holvoet T, Ceelen W, De Vos M, De Looze D
   Tijdschrift voor Geneeskunde, 2012;68:560-9

2. Fecale Transplantatie: oude wijn in nieuwe zakken
   Holvoet T, Boelens J, Van De Wiele T, Hindryckx P, Raes J, De Vos M, De Looze D
   Tijdschrift voor Geneeskunde, 2014;70:289-97

3. Ferriprieve anemie: nieuwe ontwikkelingen in diagnose en behandeling
   Holvoet T, De Vos M, Baert F
   Tijdschrift voor Geneeskunde, 2014;70:1336-1347

ORAL PRESENTATIONS

1. Soft ROCK inhibition prevents intestinal fibrosis in a murine colitis model
Presented at ECCO, February 2015, Barcelona, Spain
2. Soft ROCK inhibition prevents intestinal fibrosis in a murine colitis model
Presented at Belgian Week of Gastroenterology, February 2015, Brussels, Belgium
3. Fecal Microbiota Transplantation in Irritable Bowel Syndrom With Abdominal Bloating: Results from a prospective pilot study

Presented at Belgian Week of Gastroenterology, February 2015, Brussels Belgium
4. Transplantation de microbiote fécal chez des patients avec SII. Résultats d'une étude pilote
Presented at Journées Francophones d'Hépato-gastroentérologie et d' Oncologie digestive (JFHOD) 2015, Paris, France
5. Early Fibrostenosis in Crohn's disease is associated with multiple susceptibility loci on Immunochip analysis
Presented at ECCO, February 2017, Barecelona, Spain
6. Early Fibrostenosis in Crohn's disease is associated with multiple susceptibility loci on Immunochip analysis
Presented at the Belgian Week of Gastroenterology, February 2017, Antwerp, Belgium
Presented at ECCO, February 2017, Barecelona, Spain
7. Early Fibrostenosis in Crohn's disease is associated with multiple susceptibility loci on Immunochip analysis
Presented at the United Week of Gastroenterology, October 2017, Barcelona, Spain
8. Fecal Microbiota Transplantation in irritable bowel syndrome: results from a randomized controlled trial
Presented at the Belgian Week of Gastroenterology, February 2018, Antwerp, Belgium
9. Fecal Microbiota Transplantation in irritable bowel syndrome: results from a randomized controlled trial
To be presented at the presidential plenary session at Digestive Disease Week, June 2018, Washington DC, USA

POSTER PRESENTATIONS

1. The Effects of Radical Treatment in Patients with NSCLC
   Holvoet T, Van Meerbeeck J, Van De Wiele C, Salhi B, Derom E.
   Presented at IASLC 13th World Conference on Lung Cancer, San Francisco, USA
   Presented at European Respiratory Society Congress 2009, Vienna, Austria

2. Quantitative Perfusion Scintigraphy (QPS) or the Anatomic Segment Method (ASM) for estimating Postoperative Pulmonary Function (PF) in patients treated for non-small cel lung cancer (NSCLC): a retrospective comparison
   Holvoet T, Van Meerbeeck J, Van De Wiele C, Salhi B, Derom E.
   Presented at IASLC 13th World Conference on Lung Cancer, San Francisco, USA
   Presented at European Respiratory Society Congress 2009, Vienna, Austria

3. Soft ROCK inhibition prevents intestinal fibrosis in a murine colitis model
   Holvoet T, Devriese S, Castermans K, Boland S, Leysen D, Vandewynckel Y, Devisscher L, Vandenbossche L, Van Welden S, Dullaers M, De Rycke R, Geboes K, Bourin A, Defert O, Hindryckx P, De Vos M and Laukens D.
   Presented at DDW Congress 2015, Washington, USA; Poster of Distiction

4. Fecal Microbiota Transplantation in Irritable Bowel Syndrome With Bloating: Results from a Prospective Study
   Holvoet T, Joossens M, Wang J, Boelens J, Verhasselt B, Laukens D, Van Vlierberghe H, Hindryckx P, De Vos M, De Looze D, Raes J.
   Presented at DDW Congres 2015, Washington, USA

5. Local ROCK inhibition attenuates development of intestinal fibrosis in murine colitis
   Holvoet T, Devriese S, Castermans K, Boland S, Leysen D, Vandewynckel Y, Devisscher L, Vandenbossche L, Van Welden S, Dullaers M, De Rycke R, Geboes K, Bourin A, Defert O, Hindryckx P, De Vos M and Laukens D.
   Presented at UEGW Congress 2016, Barcelona, Spain

6. Local ROCK inhibition attenuates development of intestinal fibrosis in murine colitis
   Holvoet T, Devriese S, Castermans K, Boland S, Leysen D, Vandewynckel Y, Devisscher L, Vandenbossche L, Van Welden S, Dullaers M, De Rycke R, Geboes K, Bourin A, Defert O, Hindryckx P, De Vos M and Laukens D.
   Presented at Keystone Congress 2016, Keystone, Colorado, USA

7. Hémorrhoidectomie pédiculair par Thunderbeat. Etude piloted dans le prolapses hémorrhoidaire symptomatique. Etude de faisabilité
   De Looze D, **Holvoet T** , Laurent S, Rossler S, De Vos M
   Presented at Journées Francophones d'Hépato-gastroentérologie et d' Oncologie digestive (JFHOD) 2016

8. Early fibrostenosis in Crohn's disease is associated with multiple susceptibility loci on Immunochip analsyis
   Holvoet T, Bossuyt P, Cleynen I, De Kock I, Hindryckx P, Vermeire S, Laukens D, De Vos M
   Presented at Digestive Disease Week, May 2017, Chicago, USA

KEY NOTE LECTURES

Intestinal fibrosis in inflammatory bowel disease
Holvoet T
Presented at the World Congress on Inflammation, July 2017, London, UK

AWARDS

Best oral presentation for Early fibrostenosis in Crohn's disease is associated with multiple susceptibility loci on Immunochip analysis, presented at UEGW 2017

Onderzoeksbeurs VVGE 2015

DANKWOORD